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Abstract

Allelic loss at the short arm of chromosome 11 is one of the most common and potent events in the progression and metastasis of breast cancer. Here, we present evidence that the Integrin-Linked Kinase (*ILK*) gene maps to the commonly deleted chromosome 11p15.5 and suppresses malignant growth of human breast cancer cells both *in vitro* and *in vivo*. *ILK* is expressed in normal breast tissue but not in metastatic breast cancer cell lines or in advanced breast cancers. Transfection of wild-type *ILK* into the MDA-MB-435 mammary carcinoma cells potently suppressed their growth and invasiveness *in vitro*, and reduced the cells' ability to induce tumors and metastasize in athymic mice. Conversely, expression of the ankyrin repeat or catalytic domain mutants of *ILK* failed to suppress the growth of these cells. Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase. These findings directly demonstrate that *ILK* deficiency facilitates neoplastic growth and suggest a novel role for the *ILK* gene in tumor suppression.

Table of Contents

A. INTRODUCTION

Genetic alterations that occur in breast cancer are believed to be of importance for initiation as well as progression of the disease. These genetic alterations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, and genetic stability. The genetic abnormalities most frequently observed in breast tumors are amplification of proto-oncogenes (*MYC*, *ERBB2* and *CCND1*), mutations of *TP53*, and loss of heterozygosity (LOH) on chromosomes 3p, 6q, 7q, 8p, 9p, 11, 13q, 17, 18q and 22q (1, 2). Metastatic phenotypes have been linked to such genes as *NME1* (17q), *CDH1* (16q), *BRMS1* (11q), and *KISS1* (1q) (1, 3-5). LOH analyses have defined regions of deletion associated with metastasis on chromosomes 3p21, 15q14, 16q22 and 11p15 (2, 6)

Frequent genetic alterations on chromosome 11p15 suggest a crucial role for this region in breast (6, 7) and other adult (8-12) and childhood cancers (13-17). More recently, we have mapped two distinct regions on chromosome 11p15.5 that are subject to LOH during breast tumor progression and metastasis (6). LOH at region 1 correlated with tumors that contain ductal carcinoma *in situ* suggesting that the loss of a critical gene in this region may be responsible for early events in malignancy. LOH at region 2 correlated with a more aggressive tumor and an ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Although considerable advances have been made in the fine-mapping of chromosome 11p15.5, the tumor suppressor gene(s) encoded by this region have evaded identification.

Integrin-linked kinase (*ILK*) is an intriguing serine/threonine kinase that has been implicated in integrin-, growth-factor- and Wnt-signaling pathways (18). It binds to the cytoplasmic domains of β1 and β3 integrins and mediates the down-stream signaling events in integrin function (19). Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize (20). In *Drosophila*, the absence of *ILK* function causes defects similar to loss of integrin adhesion and *ILK* mutations cause embryonic lethality and defects in muscle attachment (21). Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene in tumor suppression has not been established. We have therefore analyzed the effect of *ILK* expression on the *in vitro* and *in vivo* tumor growth and invasion of human mammary carcinoma cells.

B. BODY

Localization of *ILK* to the LOH region on chromosome 11p15.5.

The LOH region 2 (6) extends between the markers D11S1760-D11S1331 on chromosomal band 11p15.5 (Figure-1). We constructed a 500 kb genomic contig (Karnik et al, unpublished results) that includes the critical region between D11S1760 and D11S1331. Using a PCR-based screening method, we initially isolated PAC and BAC clones that contained D11S1760 and D11S1331 markers. The order of the genomic clones in the contig was confirmed by mapping of STSs, ESTs, unigene clusters and known genes that were previously mapped to chromosome 11. Eleven novel transcripts and seven previously reported genes were PCR-mapped to the critical region between D11S1760 and D11S1331. Three of the known genes, Tata box-binding protein-associated protein (*TAF II 30*) (22), Lysosomal pepstatin insensitive protease (*CLN2*) (23) and Integrin -linked kinase (*ILK*) (24) were previously mapped only at the level of cytogenetic resolution. However, with the current mapping data, we have been able to determine the precise genomic locations of these three genes (Figure-1). The map location and its role in multiple signaling pathways makes ILK an attractive candidate tumor suppressor gene.

Loss of *ILK* expression in human breast carcinomas.

To determine whether *ILK* has a role in breast cancer progression, mRNA expression in normal and tumor breast epithelial cells was compared by Northern blot hybridization (Figure-2). A single 1.8 kb *ILK* mRNA is highly expressed in all samples of normal breast epithelial cells. Three representative examples

are shown in Figure-2 (N1, N7 and N8) In sharp contrast, there is complete loss of *ILK* mRNA expression in 9 out of 15 (~60%) invasive breast tumors and a 2-5 fold down-regulation of *ILK* mRNA in the remaining breast tumors (Figure-2A). Comparison of *ILK* mRNA expression in a panel of well-characterized breast cancer cell lines and in the non-malignant breast epithelial cell line MCF-10A is shown in Figure-2B. *ILK* mRNA expression in MCF-10A is comparable to the expression in normal breast tissue (N7, N8) (Figure-2B). However, there is a 3-5 fold down-regulation of *ILK* mRNA expression in the breast cancer cell lines MCF-7, T47D, ZR75.1, MDA-468, MDA-134, MDA-231 and MDA-435 (Figure-2B).

To further confirm these observations, *ILK* protein expression was also examined using indirect immunofluorescence microscopy in frozen samples of 20 normal and corresponding pathological human breast tissue samples. Figure-3 shows four representative examples. Immunohistochemical staining of normal breast tissue with *ILK*-specific primary antibody and rhodamine labeled secondary antibody shows specific staining of the mammary epithelial cells surrounding the lumen in normal breast tissue from breast cancer patients. *ILK* expression is particularly intense in epithelial cells both within large ducts and within terminal duct lobular units but not in the stromal compartment. Incubation with purified nonspecific rabbit immunoglobulin IgG, did not result in any positive staining of the normal epithelium of the breast (control). The normal breast tissue from four representative patients were positive, (3N, 12N, 6N and 10N) whereas ILK expression was nearly completely lost in the four corresponding infiltrating ductal carcinomas (3T, 12T, 6T, 10T) (Figure-3). These data show that *ILK* production by breast tumor cells correlates inversely with tumorigenecity and metastatic potential.

The *ILK* gene maps to chromosome 11p15.5 a region that displays a high frequency (~60%) of LOH in breast cancer. All breast tumor samples described in Figures 2 and 3 have previously been identified to contain LOH at the 11p15.5. (6). Allelic loss results in the reduction of gene dosage and thus may result in decreased expression. However, as seen in Figure-2, all tumors have LOH for 11p15.5 and yet, only some tumors show complete loss of ILK expression. Therefore, intragenic mutations or epigentic mechanisms might contribute to the biallelic silencing of the ILK gene in breast tumors. We sought to determine if mutations are involved in the dysregulation of the *ILK* gene during the progression of human breast cancer. The *ILK* gene consists of 13 exons (Melchior et al., 2000, GenBank database, GI accession AJ404847). Primers derived from the sequences flanking each exon of *ILK* were used to analyze genomic DNA from 20 invasive breast tumors and matched normal tissue from the same patients. Using PCR-single strand conformation polymorphism (PCR-SSCP), only one of the 20 tumors analyzed showed a band shift in the SSCP assay. Subsequent DNA sequencing confirmed a silent mutation at codon 352 (GCA--->GCG) (data not shown). These results demonstrate that *ILK* mRNA and protein expression is consistently down-regulated during the progression of human breast cancer and this down-regulation does not commonly involve mutations. Epigenetic mechanisms as a probable cause of *ILK* gene silencing are currently under investigation.

ILK suppresses cell growth in human breast carcinoma cells.

The inverse correlation between *ILK* expression and tumorigenecity suggested the hypothesis that elaboration of *ILK* by tumor cells into their environment may exert an inhibitory effect. To test this hypothesis, we transfected the human breast carcinoma cell line MDA-MB-435 with the *ILK* cDNA. This cell line synthesizes very low levels of *ILK* compared to normal mammary epithelial cells (Figure-2B) and can be injected into the mammary fat pad of nude mice to provide an orthotopic model system for human breast cancer tumorigenecity and metastasis. The MDA-MB-435 cells were transfected with a mammalian expression vector pIRES-EGFP containing full length *ILK* cDNA under control of the CMV promoter. A total of four stable clones expressing different levels of *ILK* have been established. Comparison of mRNA expression by Northern blot analyses revealed that the clones TR4 and TR5 expressed slightly higher levels of *ILK* mRNA compared to the clones TR2 and TR3 (Figure 4B). Based on Northern analysis, *ILK* expression in clone TR5 is 2-3 fold higher compared to the expression in the

non-malignant breast epithelial cell line MCF-10A and to the expression in normal mammary epithelial cells (Figure-2B) suggesting that ILK is overexpressed in the TR5 clone. The expression of *ILK* in empty vector controls (data not shown) is comparable to untranfected MDA-MB-435 cells (UT). *ILK* protein levels in transfected (TR5) and untransfected cellswasdetermined by indirect immunofluorescence. High levels of *ILK* protein are expressed in the transfected MDA-MB-435 cells (Figure 4Ac) compared to the untransfected control (Figure-4Ab). The *ILK* protein is localized in the cytoplasm. Most strikingly, corresponding to the low levels of *ILK* mRNA (Fig. 2B), the highly metastatic MDA-MB-435 cell line showed very little detectable *ILK* protein (Figure 4Ab).

To determine whether *ILK* overexpression had any effect on the growth properties of the MDA-MB-435 cells, we determined the growth kinetics of the clones TR3 and TR5. *ILK* expression causes the MDA-MB-435 cells to grow to a low saturation density (Figure-5A) and there is substantial growth suppression of the TR5 clone compared to untransfected MDA-MB-435 cells. The growth suppression of the transfectants was *ILK* concentration dependent with TR5 (high expressing clone) growing to a lower saturation density than TR3 (low expressing clone). Furthermore, the growth rate of TR5 was decreased by ~ 40% with a cell doubling time of 96 hours compared to the growth rate of cells transfected with vector alone or untransfected cells which had a doubling time of 48 hours.

The ability of *ILK* to suppress growth could be due to a non-specific lethal effect of protein overproduction. Alternatively, it could be a manifestation of a more specific effect on cell proliferation. To further investigate these possibilities and to establish a link between a functional *ILK* and growth suppression, we tested the growth kinetics of two *ILK* variants. *ILK* contains four ankyrin repeats at the NH2-terminus (18) that participate in protein-protein interactions important for integrin-, growth-factor- and Wnt- mediated signaling. First, a deletion mutant, Δ ANK lacking this domain was constructed. In addition, the residue E359 has been shown to be essential for *ILK* function (18). We therefore constructed an *ILK* point mutant (E359K) in which the highly conserved Glu359 within the *ILK* catalytic domain was substituted with lysine. The growth rates of the stably transfected *ILK* mutant clones Δ ANK and E359K compared to the *ILK* transfectant TR5 are shown in Figure 5B. As discussed above, overexpression of the wild-type *ILK* strongly inhibited growth of the MDA-MB-435 cells. In contrast, both the Δ ANK and E359K mutants lost their capacity to suppress the growth of the MDA-MB-435 cells (Figure-5B) arguing against a non-specific effect of protein overproduction.

Expression of *ILK* in MDA-MB-435 cells leads to a G1 cell cycle arrest.

The observed growth suppression by *ILK* could be caused by either increased apoptosis or inhibition of cell proliferation. To investigate the mechanisms underlying the growth suppression by *ILK* expression, we studied apoptosis by fluorescence-activated cell sorting (FACS) analysis of Annexin-V stained *ILK* and vector transfectants. There was no increase in the rate of apoptosis in *ILK*-expressing cells compared to vector transfectants (data not shown). Therefore, programmed cell death does not seem to account for the growth suppression of *ILK* transfected cells.

To test for cell cycle regulation by ILK, propidium iodide stained MDA-MB-435 clones were analyzed by flow cytometry. Expression of ILK increased the number of cells in G0/G1 from 64 to 85% (Figure-5C, VT and TR5-ILK) and decreased inversely the number of cells in S and G2/M phase from 26 and 10% to 9 and 5% (Figure-5C, VT and TR5-ILK). In contrast, the cell cycle profiles of the two ILK variants Δ ANK and E359K were very similar to the parental MDA-MB-435 cells. These results indicate that ILK growth suppression results from G1 cell cycle arrest. The accumulation of cells in the G0/G1 phase of the cell cycle suggests arrest predominantly at the G1/S boundary. ILK overexpression does not induce cell death or apoptosis but induces a very pronounced growth arrest with 85% of the cells in G0/G1, a property that is the hallmark of growth/tumor suppressors. Growth suppressor genes play an important role in checkpoint function and loss of genes associated with checkpoint functions seem to have important implications in the development of cancer. The percentage G1 arrest induced by *ILK* is

comparable to the effect on cell cycle progression induced by the *p21* cyclin-dependent kinase inhibitor (25).

ILK suppresses the invasive phenotype of human breast carcinoma cells.

The invasiveness of tumor cells represents one of several important properties necessary for the formation of metastases. Cell migration on vitronectin in vitro has been linked to the metastatic capacity of tumor cells in vivo (26, 27). To examine the effects of *ILK* expression on breast cancer cell invasion, the ability of vector and *ILK* transfected MDA-MB-435 cells to degrade and invade vitronectin -coated polycarbonate membrane was investigated. As shown in Figure-6A, a significant reduction in invasive potential was noted in the ILK expressing clone TR5 (ILK) compared to vector transfected MDA-MB-435 cells (VT) (Figure-6A). Cell invasion through membranes coated with vitronectin, is decreased by 60% in MDA-MB-435 cells expressing *ILK* compared to vector transfected MDA-MB-435 cells. In contrast, the two ILK variants Δ ANK and E359K have no significant effect on cell invasion under identical conditions (Figure-6A). In fact, there is a slight increase in invasive potential of the variant clones (Δ ANK and E359K), suggesting a dominant-negative effect, perhaps due to inhibition of endogenous ILK in the MDA-MB-435 cells. These results indicate that *ILK* expression abates extracellular matrix invasion of tumor cells *in vitro*, one of the hallmarks of tumorigenecity and transformed cell growth.

Cell adhesion, migration and invasion are controlled by the levels of integrins and by the amount of fibronectin matrix around the cell (20). Because the αvβ3 and α5ß1 integrins have been implicated in the regulation of angiogenesis, tumor cell migration, invasion and metastasis, we speculated that *ILK* might regulate cell migration via alteration of the cellular composition of integrins. Using a panel of specific antibodies against these integrins in flow cytometry analysis, we compared integrin expression patterns in relation to the *ILK* expression status. The results are shown in Figure-6B. The *ILK* transfected cells demonstrated a 22% increase in levels of the growth-suppressing integrin α5β1 and a 31% decrease in levels of the growth-promoting integrin αvβ3 compared to the control cells. The changes in levels of αvβ3 and α5ß1 expression in *ILK* transfected cells although relatively moderate in comparison to control cells, nonetheless, were highly significant. Collectively, these observations suggest that *ILK* reduces the invasive potential of MDA-MB-435 cells by altering their integrin profiles, which changes their ability to perceive and interact with their extracellular environment.

ILK suppresses tumor formation and metastasis in nude mice.

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet not all of the cellular growth properties commonly associated with the cellular state *in vitro* are required for neoplastic growth *in vivo* and vice versa. Therefore, loss of tumorigenecity under expression of *ILK in vivo* would be a critical test to substantiate the growth suppressor function of *ILK*. The mammary carcinoma cell line MDA-MB-435 forms tumors at the site of orthotopic injection, metastasizes in nude mice and closely resembles the course of human breast cancer (52). To investigate whether *ILK* expression affected tumor formation in nude mice, two different *ILK* transfectant clones (TR5-ILK and TR3-ILK) and two vector controls were inoculated into the subaxillary mammary fat pads of 4-6 week old athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. All MDA-MB-435 vector transfectants were already palpable 7 days after injection. Subsequently, the tumors of vector transfectants grew steadily attaining mean volumes of 3.0 cm^3 (mean $\pm$ s.d.) at 15 weeks (Fig. 7A and B). In contrast, only 2 of 12 mice injected with ILK transfectants developed tumors. The tumor growth of ILK transfectants was significantly slower than than that of control transfectants ($P <$ 0.005, Fisher variance analysis). At sacrifice, (15 weeks) the *ILK* tumors reached a mean volume of only 0.45 cm^3 (mean $\pm$ s.d.) which was significantly smaller than control tumors ($P < 0.001$, Student's *t*-test). Vector transfected MDA-MB-435 cells developed an average of 12-24 lung metastases per mouse

(Figure-7C). Additional tumor masses were present in central venous blood vessels, the diaphragm, and lymph nodes of vector transfectants (data not shown). In contrast, with the ILK transfectants, only one of the two animals that developed tumors showed a single metastatic colony in the lung. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). These results clearly demonstrate that the expression of *ILK* in human MDA-MB-435 breast carcinoma cells significantly suppresses tumorigenecity and metastatic ability in athymic nude mice.

C. CONCLUSIONS

Growth inhibitory functions of *ILK*.

The present study reveals that expression of *ILK* potently suppresses the growth and tumorigenecity of the human mammary carcinoma cells MDA-MB-435 *in vitro* and *in vivo*. This growth suppression activity requires a functional *ILK* protein, since expression of wild-type *ILK*, but not the ankyrin repeat or the catalytic domain mutants, resulted in growth suppression of MDA-MB-435 cells. The demonstration of a growth suppressive function suggests a possible role for *ILK* in tumor suppression and directly implicates its loss in processes regulating the growth and maintenance of the malignant phenotype in human breast cancer. Our results suggest that the growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression at G1 phase. During this process, the neoplastic cells cease to proliferate and lose their ability to migrate through vitronectin membranes and to induce tumor growth and metastasis in nude mice. Our results are consistent with earlier micro-cell mediated chromosome transfer experiments showing that introduction of human chromosome 11 into MDA-MB-435 cells suppressed metastasis in immunocompromised animals (28).

ILK seems to play a dual role in the MDA-MB-435 model system. First, it regulates cell-cycle progression at the G1/S boundary and second, it modulates the levels of integrins, transmembrane receptors that have been shown to regulate cell growth, survival, and differentiation. Growth suppressor genes play an important role in checkpoint function and silencing of genes associated with checkpoint functions seem to have important implications in the development of cancer. Integrin signals are necessary for cells to traverse the cell division cycle. Progression through the G1 phase of the cell cycle requires the sequential activation of the cyclin-dependent kinases *(Cdk's) Cdk 4/6 and Cdk 2* and the activities of these kinases are regulated by integrins (29). In view of our observation that *ILK* regulates cell-cycle progression at the G1 phase, it is quite probable that the integrin interactions with the *Cdk's* are mediated by *ILK*. *ILK* could interact with specific integrin cytoplasmic domains and couple them to appropriate downstream signaling pathways. This in turn could regulate such functions as coordination of growth factor signals and altering gene expression required for cell proliferation and differentiation.

The interaction of cells with the surrounding extracellular matrix (ECM) affects many aspects of cell behavior, including the migratory properties of cells, their growth, and differentiation (29). Integrins are transmembrane heterodimeric proteins that mediate such interactions. The large extracellular part of both α and ß subunits bind proteins within the ECM. The short cytoplasmic domain of the β integrin subunit anchors the cytoskeleton to the plasma membrane via intermediary adaptor proteins. In *Drosophila* (21), *ILK* has been shown to be a component of the structure linking the cytoskeleton and plasma membrane at sites of integrin-mediated adhesion. The absence of *ILK* function in *Drosophila* causes defects similar to loss of integrin adhesion. Similarly, the downregulation of *ILK* expression in mammary epithelial cells could cause the cells to become more invasive. Indeed, as seen in our present study, *ILK* overexpression in the highly metastatic breast cancer cell line MDA-MB-435 causes the cells to lose their tumorigenecity and metastatic potential. What is the biological significance of *ILK*-mediated regulation of the α5ß1 and αvβ3 integrins? Previous studies have shown that α5ß1 expression is frequently lost during malignant progression, a phenomenon that has been observed in human colonic, mammary and pancreatic cancer (30,31). Expression of the α5β1 integrin in HT29 human colon carcinoma cells also blocks tumorigenecity in nude mice (32). In contrast, the αvβ3 integrin cooperates with certain growth factors, potentiating their effects on cells and its expression correlates with a role in metastasis. Indeed,

expression of integrin αvβ3 is significantly higher in breast tumors of patients with metastases than in those without metastasis and may have a role in skeletal metastases (33). Therefore, our observation that *ILK* modulates the levels of α5ß1 and αvβ3 integrins is very significant and suggests that *ILK* may reduce the invasive potential of MDA-MB-435 cells by altering their integrin profiles.

Frequent down-regulation and lack of mutations of the ILK gene in breast carcinoma.
We determined by Northern blot and immunohistochemical analysis that most invasive breast carcinomas exhibit complete loss or very low expression of *ILK* mRNA and protein. However, in our present study, we detected no homozygous deletions or intragenic mutations in the *ILK* gene. Thus, it is likely that the *ILK* gene is not a target for mutations in many cancers, and other mechanisms for *ILK* down-regulation should be considered. *ILK* maps to chromosome 11p15.5, a region that exhibits a high frequency (40-60%) of LOH in breast and other adult and childhood tumors (6,13). It is thought that LOH alone cannot completely suppress *ILK* expression, as many genes can be expressed monoallelically (34,35). Although all breast tumors used in this study were previously described (6) to have LOH at 11p15.5, a small number of breast tumors still express *ILK* suggesting that *ILK* can be expressed monoallelically. Biallelic inactivation of the *ILK* gene could result either from epigenetic inactivation of both parental alleles or from epigenetic modification of one allele and loss of the second allele via mechanisms that result in LOH. Indeed, the *p16/CDKN2* and the *p15INK4B* cell cycle regulator genes are located at a region of high LOH on chromosome 9p21 and individual alleles in neoplasia are selectively silenced by promoter hypermethylation (36,37). While the *Maspin* tumor suppressor gene is biallelically inactivated by aberrant cytosine methylation and heterochromatinization of the promoter (38), the down-regulation of the *KAI1* metastasis gene involves neither mutations nor promoter hypermethylation. (39,40). It has been suggested that there is a group of tumor suppressor genes that are unrecognized because the primary mechanism for their silencing is not known. Such genes may affect the cancer cell phenotype by expression changes and have been classified as Class II tumor suppressor genes (41). The molecular basis for the down-regulation of the *ILK* tumor suppressor gene in breast cancer is currently under investigation. The loss of expression that occurs during malignant progression of primary breast tumors suggests that *ILK* has potential value as a prognostic marker. Future studies should test the prognostic value of *ILK* on a larger scale, in order to establish more firmly a correlation between loss of *ILK* expression and progression of breast and other cancers.

The paradoxical effect of *ILK* on tumorigenecity.
Previous studies have shown that *ILK* overexpression results in loss of cell-cell adhesion (42), promotes suppression of anoikis by activation of *PKB/Akt* signaling (43) and oncogenic transformation of the rat intestinal epithelial cells by activation of the *LEF-1/ß-catenin* signaling pathways (44,45). In contrast, recent studies in *Drosophila* (21) have shown that *ILK* is required for integrin-mediated adhesion, but not for signaling involving *ß-catenin (armadillo)* or *PKB. ILK* mutations in *Drosophila* cause embryonic lethality and defects in muscle attachment, and clones of cells lacking ILK in the adult wing fail to adhere, forming wing blisters. The *ILK* coding sequence is highly conserved in different species (21), suggesting that it has an essential biological function in evolution. We have shown that transfection of the MDA-MB-435 mammary carcinoma cells with the *ILK* gene reduced the cells' ability to induce tumors and to invade through vitronectin membranes *in vitro*. The down-regulation of *ILK* in metastatic breast cancer cell lines and invasive breast tumors strongly suggests that *ILK* might block uncontrolled cell growth in normal breast tissue and that its absence may be permissive for malignant tumor growth. The negative correlation between *ILK* expression and growth suppression is unexpected when considered with the current concept that kinases are positively associated with tumorigenesis (for example, c-erB2). However, Lynch et al.(46) have demonstrated that *ILK* is not a typical protein kinase and lacks a DFG motif or a conserved substitute for the catalytic aspartate residue found in other kinases and they and other investigators (47) have failed to detect protein kinase activity in *ILK* immunoprecipitates. Mutations in the kinase domain shown to inactivate the kinase activity of human *ILK* do not show any phenotype in

Drosophila (21), suggesting a kinase independent function for *ILK*. Thus, it is likely that the functions of ILK are more complex than previously envisioned; and the divergent and often paradoxical effects mediated by *ILK* may depend on the particular cell-type, the cell-specific integrins that are activated by a cell, and on whether the adaptor protein *ILK* activates a serine-473 kinase or phosphatase.

In conclusion, we have shown that the loss of *ILK* expression is associated with the acquisition of a malignant breast tumor phenotype and that *ILK* may directly act as a tumor suppressor, presumably by controlling cell division. The absence of the *ILK* tumor suppressor protein, may promote uncoordinated G1 cell cycle progression, allowing cells to bypass the normal signaling processes regulated by growth factors and cell anchorage, leading to tumorigenesis. This novel information regarding the biological effects of *ILK* provides hopeful therapeutic utility for this potent tumor suppressor gene in the management of breast cancer.

D. MATERIALS AND METHODS

Mapping of *ILK* to LOH region 2.

Two YAC clones (847a12 and 696H10), a BAC clone (BAC 1760) and a PAC clone (PAC1331) overlapped the LOH region 2 and were used to construct a genomic contig across this the ~336 kb LOH region 2 (Karnik et al, unpublished results). The contig was assembled by mapping of STSs and polymorphic markers. *ILK* was mapped to this region using the primers (5'-TGGAACCCTGAACAAACACT-3' and 5'-AGTCCCTGCTCTTCCTTGTA-3').

Cell lines and cell cultures.

MDA-MB-435 is an estrogen receptor-and progesterone receptor-negative, metastatic, ductal human breast carcinoma cell line derived from a 31 year old female (48). It has a heterogeneous chromosome complement of between 50 and 58 chromosomes, including several derivative marker chromosomes. The cell line readily forms tumors when injected into mammary fat pads (mfp) of nude mice and macroscopic metastases to lungs and regional lymph nodes can be identified 15-18 weeks postinoculation. This pattern closely parallels clinical observations. Therefore, this cell line was chosen for our studies. MDA-MB-435 cells were the generous gift of Dr. Janet Price (University of Texas M.D. Anderson Cancer Center, Houston, TX). Human breast cancer cells MCF-7, T47D, MDA-MB-231, ZR75-1, MDA-MB-134, MDA-MB-468 were obtained from the American Type Culture Collection (Rockville, Maryland). All human breast cancer cell lines were maintained in DMEM-F12 medium supplemented with 10% fetal calf serum and no antibiotics. The neomycin resistant ILK transfectants were maintained in DMEM-F12 containing 300 μg/ml G418 (Life Technologies Inc., Gaithersburg, MD). All cell lines were free of *Mycoplasma* sp. contamination as determined by a PCR-based test (Pan Vera, Madison, WI).

Northern blotting and immunohistochemistry.

Primary tumor and adjacent normal breast tissue samples were obtained from 20 randomly selected breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). Samples of these tumors and corresponding non-involved tissue from each patient were collected at the time of surgery, snap-frozen, and transferred to -80°C. Clinical and histopathological features of the tumors was performed by the Pathology Department at CCF. An initial cryostat section was stained with H and E stain to determine the proportion of contaminating normal tissue in the tumor.

Total RNA from cell cultures and tumor tissues was was isolated using a RNeasy Kit® (Qiagen, Chatsworth, CA). Total RNA (20 μg) was size-fractionated in a 1% agarose gel containing 2.2 M formaldehyde. After transfer and UV-cross-linking, the nylon membranes were probed with a full-length ^{32}P labeled *ILK* cDNA. All the immunohistochemical determinations were performed on representative samples snap-frozen in liquid nitrogen and stored at -80°C until sectioning. Cryostat sections (4-6 μm thick) of tumor blocks were deparaffinized with xylene, rehydrated, and microwaved for 10 min. in 10 mM citrate buffer (pH 6.0). MDA-MB-435 cells were grown on sterile microscope cover-slips

(Fisherbrand). The cover-slips with MDA-MB-435 cells and slides containing tissue sections were fixed in 4%paraformaldehyde solution for 12 min., washed in PBS, pre-incubated with 10% Triton X-100 in PBS and incubated at room-temperature with the rabbit polyclonal IgG Anti-*ILK* (Upstate Biotechnology, Lake Placid, NY) for 1 h. After being washed with PBS, bound antibodies were visualized using rhodamine conjugated goat anti-rabbit IgG secondary antibody. Sections were visualized by fluorescence microscopy. The optimal concentration of primary and secondary antibody was determined by titration and ranged from 1:50-1:200. For negative controls, in all instances, we used non-specific IgG as the primary antibody.

Construction and transfection of wild-type and mutant forms of *ILK*.
A partial *ILK* cDNA clone (EST bb36h09.y1 from GenBank) was used to obtain a full length clone from a human placental cDNA library and its identity confirmed by sequence analysis and database comparison. The *ILK* catalytic domain mutant E359K was created as described earlier (42). Briefly, mutations were introduced into wild type *ILK* cDNA with the Promega Altered Sites II (Promega, WI). A mutant oligomer (with the altered nucleotide underlined) was used to change glutamic acid at position 359 to lysine (E359---> K, 5'-CTGCAGAGCTTTGGGGGCTACCCAGGCAGGTG-3'). To create the ankyrin repeat deletion mutant Δ ANK (residues 165-451), the 858 bp fragment of *ILK* cDNA lacking the NH2-terminal ankyrin repeat domain was PCR amplified from the wild type *ILK* cDNA. The mutant clones were confirmed by dideoxy sequencing. The full-length and mutant *ILK* cDNA inserts were cloned in the eukaryotic expression vector pIRES2-EGFP (Clontech. Laboratory, Inc.). MDA-MB-435 cells were transfected with plasmid vector alone or containing the cDNA for *ILK* using LipofectAMINE reagent (Life Technologies Inc.). Briefly, cells were grown to ~60% confluence in 6-cm tissue culture dishes, rinsed twice with serum-free medium, overlaid with a mixture of 5 μg of DNA and 10 μl of LipofectAMINE reagent diluted in serum-free medium, and incubated at 37°C in 5% CO_2/95% air for 18 h. The transfection medium was then replaced with fresh medium, and 36 h later, the cells were harvested, diluted in growth medium containing 500 μg/ml G418 and split 1:30 for the selection and establishment of clonal cell lines.

Cell growth rate, cell-cycle analysis and apoptosis assay.
For growth rate analysis, untransfected, vector transfected and *ILK* transfected cells were plated at a density of $2x10^5$ in DMEM -F12 plus 5% FBS medium in 24 well cluster plates. Viable and dead cells were assessed by counting with trypan blue exclusion at day 2, day 4, day 6 and day 8. Stable *ILK* transfectants or control cells were trypsinized and the cell numbers per dish were measured by Coulter counting and also using a hemocytometer.

Flow cytometry was used to determine the cell cycle distributions as described (25). *ILK* transfected and parental MDA-MB-435 cells were washed with PBS twice then fixed in 70% ethanol for 30 minutes at 4°C. The cells were treated with 1Ú DNase-free RNase in 1ml of PBS for 30 minutes at 37°C, and finally, resuspended in 0.05 mg ml^{-1} propidium iodide (made as a 10X stock in PBS) and cells were analyzed by flow cytometry using a FACScan model (Becton Dickinson). Ten thousand forward scatter gated events were collected for each sample. Fluorescence measurements were accumulated to form a distribution curve of DNA content. Fluorescence events due to debris were substracted before analysis.

The degree of apoptosis in vector control and *ILK* transfected MDA-MB-435 cells was quantified by using a commercially available kit with fluorescein-labeled annexin V (49) (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. Samples were analyzed on a Becton Dickison FACScan flow cytometer (a generous gift from Keck Foundation).

Cell migration assay.
Cell migration assays were performed as described earlier(50) using modified Boyden chambers (tissue culture-treated, 6.5 mm diameter, 10-μm thickness, 8-μm pores, Transwell®; Costar Corp., Cambridge, MA) containing polycarbonate membranes coated on the underside of the membrane with 10 μg/ml vitronectin in PBS. The *ILK* transfected and control cells were harvested with 0.05% Trypsin-EDTA, washed twice with quenching medium (serum free medium containing 5% BSA), and then resuspended in quenching medium (10^6 cells/ml). About 50,000- 100,000 cells were then added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for 6 h at 37°C in a CO_2 incubator. The nonmigratory cells on the upper membrane were removed witha cotton swab, and the migratory cells attached to the bottom surface of the membrane were washed with PBS, extracted with 300ul extraction buffer and absorbance determined at 560nm. All values have had background substracted, which represents cell migration on membranes coated with BSA (1%). Each determination represents the average of three individual wells, and error bars represent the standard deviation (SD).

Analysis of cell surface integrin profiles.
Fluorescence-activated cell analysis (51) was used to identify the integrin profiles on MDA-MB-435 cells in response to *ILK* expression. Monolayer cultures (60-80% confluency) ILK transfected and control cells were trypsinized and washed in culture medium. Briefly, harvested cellswere divided into equal aliquots of $2.5x10^5$ cells/ml in serum free medium plus 1% BSA. After two washes in this medium the cells were resuspended in 1:50 dilution of anti- $\alpha v\beta 3$ or $\alpha 5\beta 1$ specific antibody (Chemicon)in serum-free medium plus 1% BSA for 1h on ice. After two washes in serum-free medium plus 1% BSA, the cells were incubated in 1:100dilution of $F(ab')_2$ secondary anti-goat antibody conjugated with FITC (ICN Biomedicals) in this same medium for 1h on ice. The cells were washed twice in PBS/ 0.1%BSA and resuspended in the same solution. These samples were then analyzed using a Becton Dickinson FACScan and the data analyzed using the CellQuest software.

Tumorigenecity and metastasis assays.
Cells (10^6) were injected into the subaxillary mammary fat pads of 4-6 week-old female athymic nude mice Ncr nu/nu (10-12 mice/group; Taconic Labs, Germantown, NY) as described (52). Mice were maintained under the guidelines of NIH and the Cleveland Clinic Foundation. All protocols were approved and monitered by the the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. Tumors were monitered weekly after inoculation. When the mean tumor diameter reached 1.0-1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthetic. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastases.

E. KEY RESEARCH ACCOMPLISHMENTS:
- Chromosome 11 harbors a breast cancer metastasis suppressor gene
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the growth of the metastatic breast cancer cell line MDA-MB-435 both in vitro and in vivo.

These data suggest that ILK functions as a metastasis suppressor gene in breast cancer

People Receiving Salaries From Award:
Ping Chen, Wei-Zhen Shen, Pratima Karnik

F. REPORTABLE OUTCOMES:

- ILK expressing breast cancer cell lines
- Nude mouse model of breast cancer with and without ILK expression
- Chen P, Shen W-Z, and **Karnik,P.** Suppression of Malignant Growth of Human Breast Cancer Cells by Ectopic Expression of Integrin-Linked Kinase. *International Journal of Cancer* (Oct 10th 2004). Preprint included in Appendix
- **Karnik P**, Chen P, Tidwell N and Shen W-Z. *Integrin-Linked Kinase* Suppresses Malignant Growth of Human Breast Cancer Cells. Cancer Genetics and Tumor Suppressor Genes Meeting, Cold Spring Harbor, 2002
- **Karnik P**, Chen P, Tidwell N and Shen W-Z. Identification of breast cancer associated genes on chromosome 11. Cancer Genetics and Tumor Suppressor Genes Meeting, Cold Spring Harbor, 2000
- **Karnik P**, Chen P, Tidwell N and Shen W-Z. Suppression of Malignant Growth of Human Breast Cancer Cells by ectopic expression of *Integrin-Linked Kinase* "Era of Hope", Department of Defense Breast Cancer Research Program Meeting, August 2002, Orlando, Florida

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H. FIGURE LEGENDS:

Figure-1: Localization of *ILK* gene to the tumor suppressor region (LOH region 2) on chromosome 11p15.5. A transcript map of the LOH region is schematically represented with the relative location of the polymorphic markers, known genes, unigene clusters and expressed sequence tags (EST's).

Figure-2: Northern blot analysis of *ILK* mRNA expression. (A) Total RNA was isolated from normal breast tissue (N1, N7, N8), and fifteen invasive breast tumors (T1 to T15). B) Total RNA from exponentially growing non-malignant (MCF-10A), non-metastatic (MCF-7, T47D, ZR75.1, MDA-468 and MDA-134) and metastatic (MDA-435, MDA-231) breast cancer cell lines and normal breast tissue (N7, N8) was hybridized with ^{32}P-labeled *ILK* probe. Hybridization with the β-actin probe serves as control.

Figure-3: Immunohistochemical detection of *ILK* expression in normal breast tissues (3N, 12N, 6N, 10N) and corresponding invasive ductal carcinomas (3T, 12T, 6T, 10T). Normal ducts were positive for *ILK* expression whereas the invasive ductal carcinomas expressed little or no *ILK*. Control-Normal tissue minus primary antibody.

Figure-4: MDA-MB-435 cells were transfected with pIRES-EGFP vector containing full length *ILK* cDNA and four stable clones were isolated. (A) Immunohistochemical analysis of *ILK* expression in the MDA-MB-435 cells (b) before and (c) after transfection (stable clone TR5-*ILK*) (a) no primary antibody control. (B) Northern blot analysis of parental (UT) and *ILK* transfected MDA-MB-435 cells. TR2, 3, 4 and 5 represent stable *ILK* expressing clones. mRNA expression was determined by hybridization with ^{32}P-labeled *ILK* probe. β-actin expression serves as control.

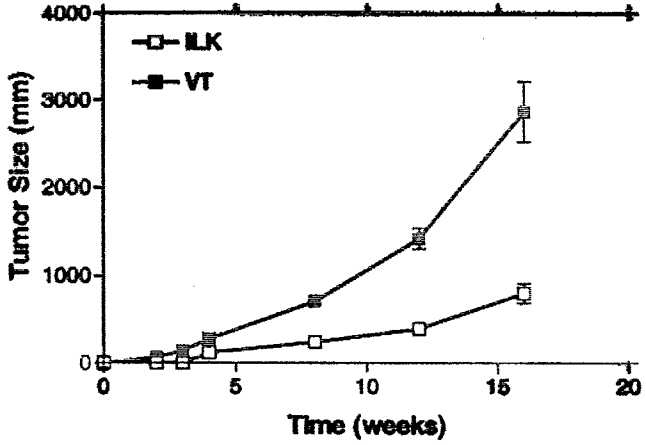
Figure-5: Growth effects of wild type and mutant alleles of *ILK* in MDA-MB-435 breast cancer cells. The MDA-MB-435 cells were transfected with either full-length *ILK* cDNA, *ILK* mutant ΔANK, *ILK* mutant E359K or eukaryotic expression vector and stable clones were obtained. (A) Growth rates of two stable *ILK* expressing MDA-MB-435 cell clones (TR3-*ILK* and TR5-*ILK*) that contain full length *ILK* cDNA compared with a stable clone containing empty vector (VT) and untransfected MDA-MB-435 cells (UT). (B) Growth rates of *ILK* mutants ΔANK and E359K compared with the wild type *ILK* expressing clone TR5-*ILK*. The means of three independent experiments are shown. Bars represent SE. (C) Cell-cycle analysis by propidium iodide staining in MDA-MB-435 cells (UT), transfected with empty vector (VT), with full length *ILK* cDNA (TR5-*ILK*) or with the *ILK* mutants ΔANK and E359K. The regions between the vertical lines from left to right represent cells in G0/G1, S and G2/M respectively.

Figure-6: Cell invasion assay of MDA-MB-435 cells transfected with vector (VT), full length *ILK* and its variants (ΔANK, E359K). Cell invasion through vitronectin was analyzed using a modified Boyden chamber. Cells that invaded to the lower surface of the membrane were lyzed and absorbance determined at 560 nm. (B) Flow cytometric analysis of α5β1 and αvβ3 integrins expressed on the surface of *ILK* transfected and parental MDA-MB-435 cells. The relative fluorescence intensity of cells stained with α5β1 and αvβ3 antibodies is represented as percentage of cell shift. Bars represent S.E.

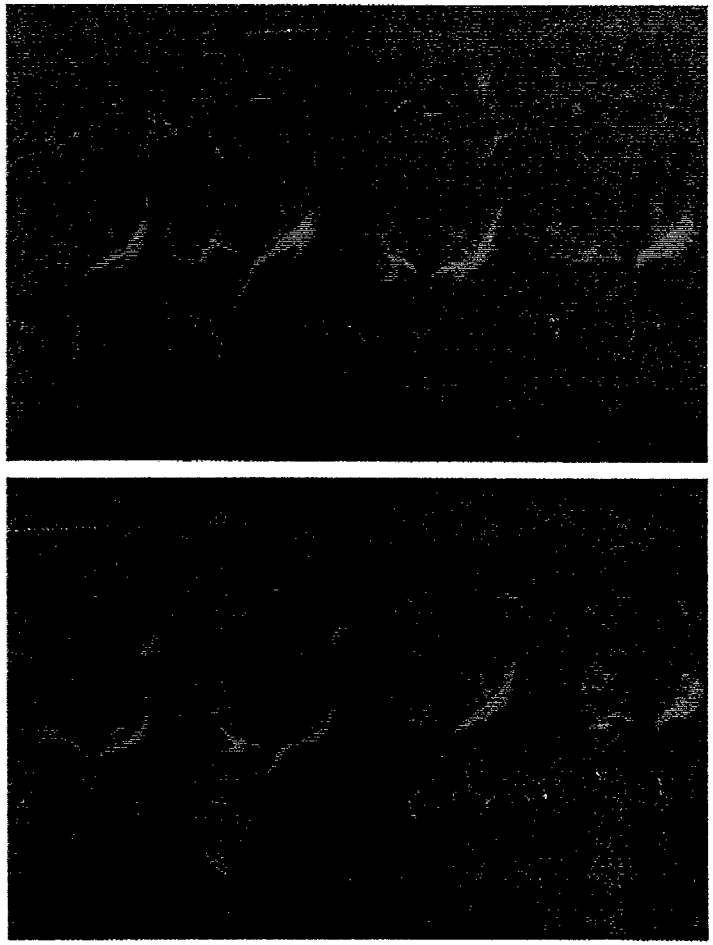
Figure-7: (A) In vivo tumor growth of *ILK* transfected (-) and vector transfected ( ) MDA-MB-435 cells in mammary fat pads of athymic nude mice. Each point represents the mean + SE of tumors. (B) Five x 10^5 cells of *ILK* transfected (top panel) or vector transfected (bottom panel) MDA-MB-435 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 15 weeks at which time the mice were photographed and sacrificed. (C) Lung colony formation in athymic nude mice injected with vector transfected (VT) or *ILK* transfected *(ILK)* MDA-MB-435 cells. Bars represent S.E.

APPENDIX

A.


4000
3000
2000
1000
0
ILK
VT
Tumor Size (mm)
0
5
10
15
20
Time (weeks)

B.



C.

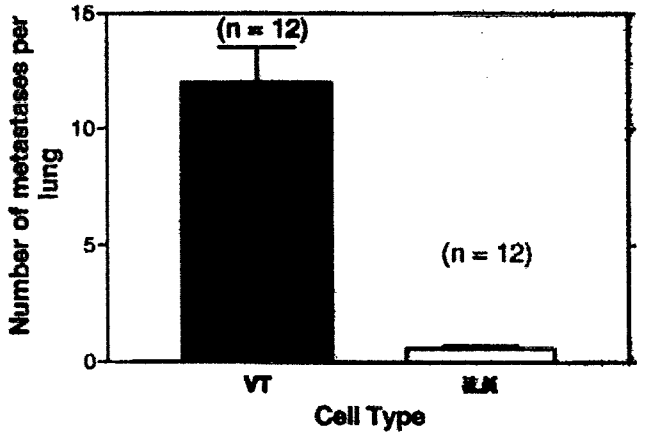

15
10
5
0
(n = 12)
(n = 12)
Number of metastases per lung
VT
ILK
Cell Type

FIGURE 1

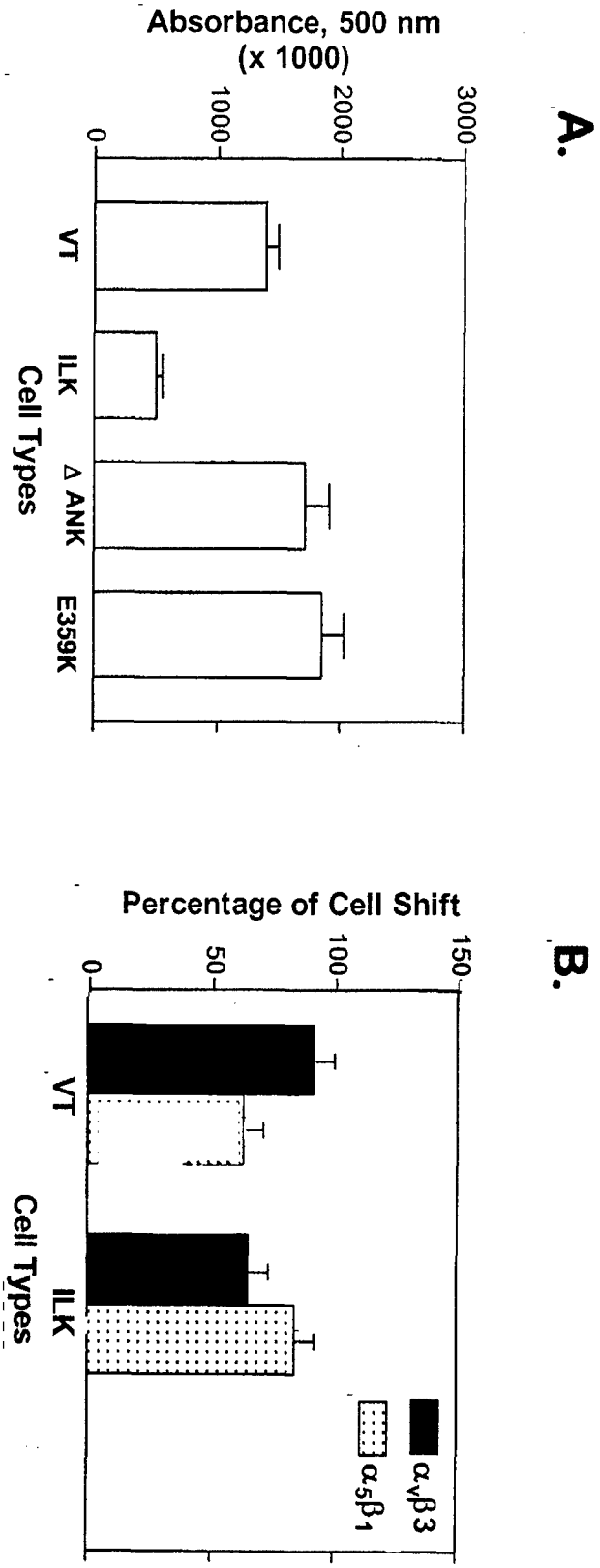

A.
Absorbance, 500 nm
(x 1000)
0
1000
2000
3000
VT
ILK
Δ ANK
E359K
Cell Types
B.
Percentage of Cell Shift
0
50
100
150
VT
ILK
Cell Types
$\alpha_v\beta_3$
$\alpha_5\beta_1$

FIGURE 2

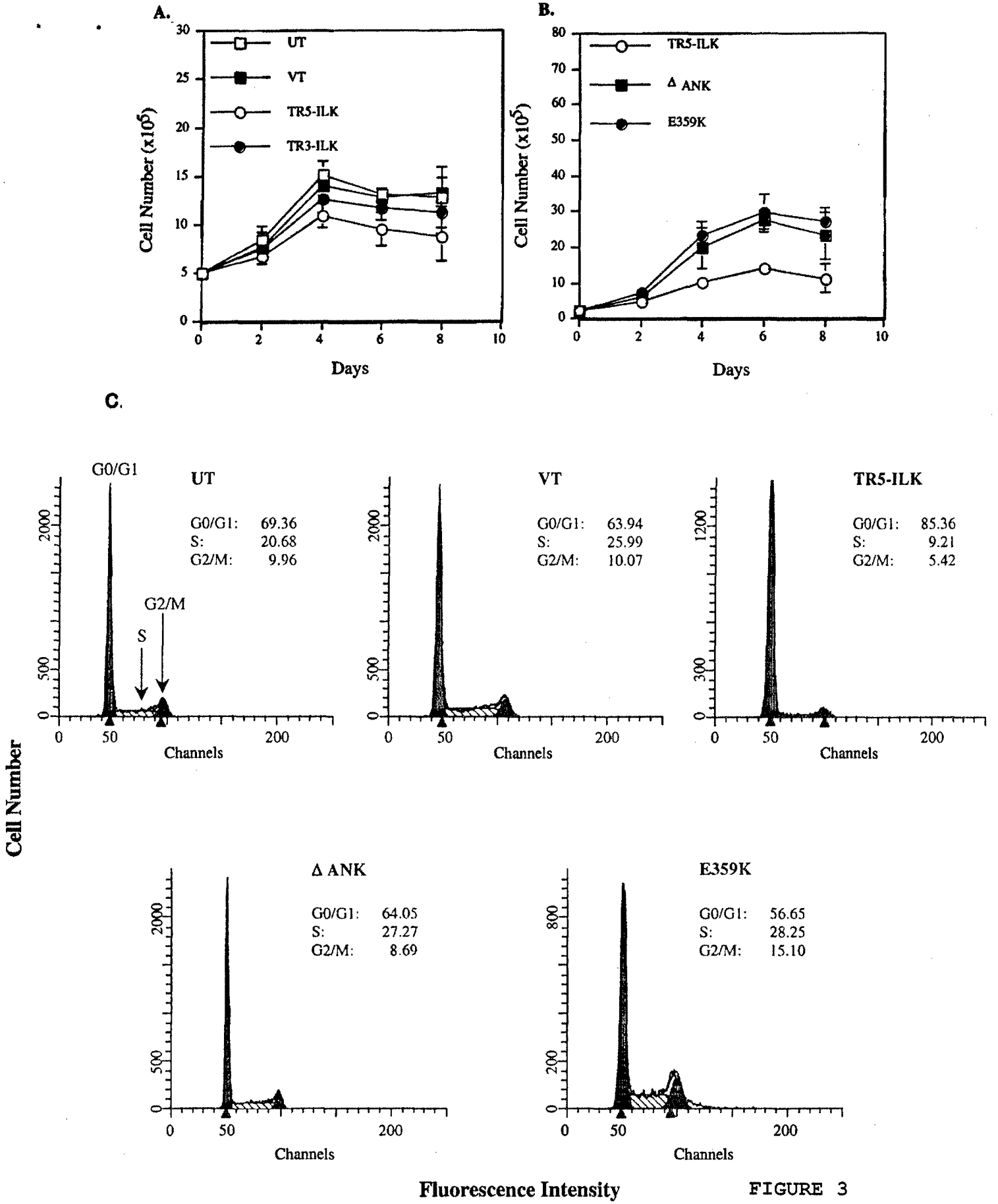
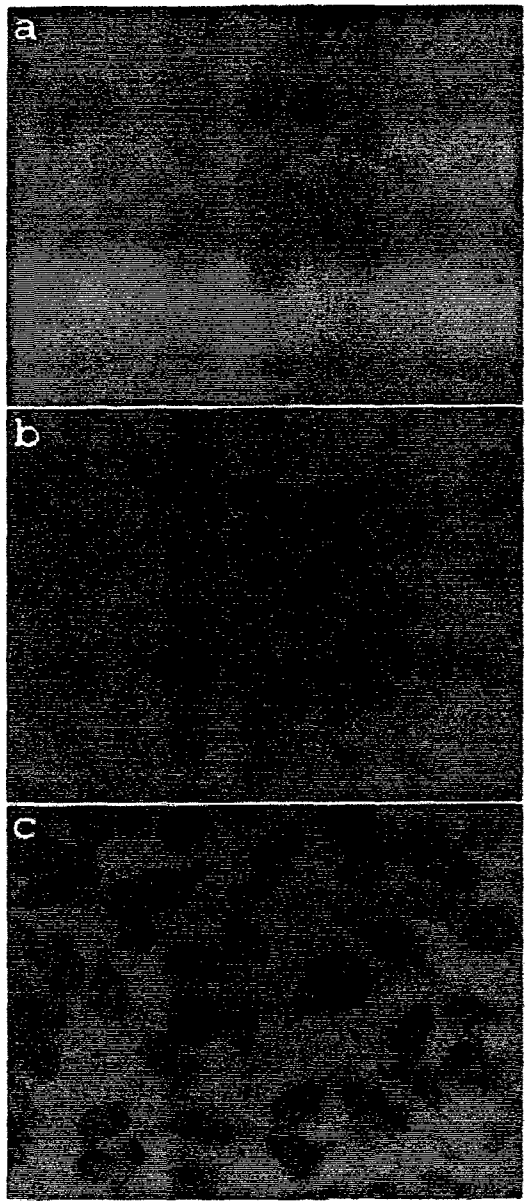

A.
UT
VT
TR5-ILK
TR3-ILK
Cell Number (x10^5)
Days
B.
TR5-ILK
Δ ANK
E359K
Cell Number (x10^5)
Days
C.
G0/G1
S
G2/M
UT
G0/G1: 69.36
S: 20.68
G2/M: 9.96
VT
G0/G1: 63.94
S: 25.99
G2/M: 10.07
TR5-ILK
G0/G1: 85.36
S: 9.21
G2/M: 5.42
Δ ANK
G0/G1: 64.05
S: 27.27
G2/M: 8.69
E359K
G0/G1: 56.65
S: 28.25
G2/M: 15.10
Channels
Cell Number
Fluorescence Intensity

FIGURE 3

A.



B.

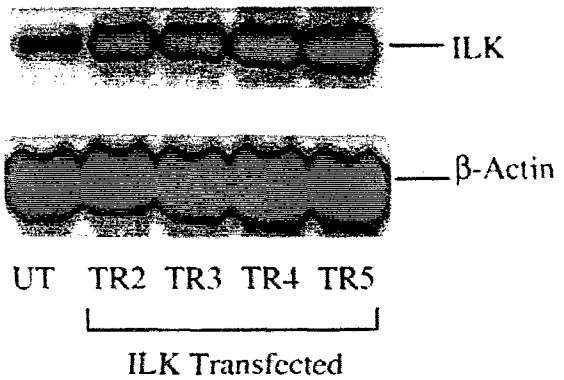


FIGURE 4

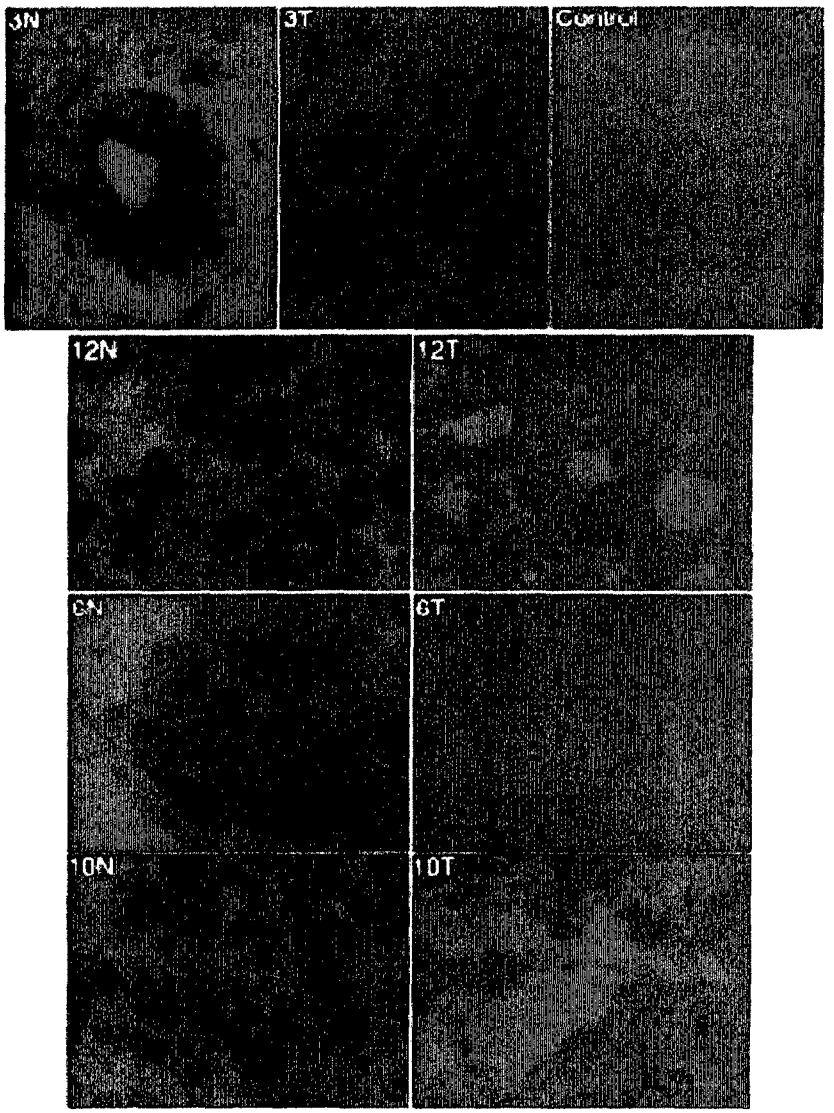


FIGURE 5

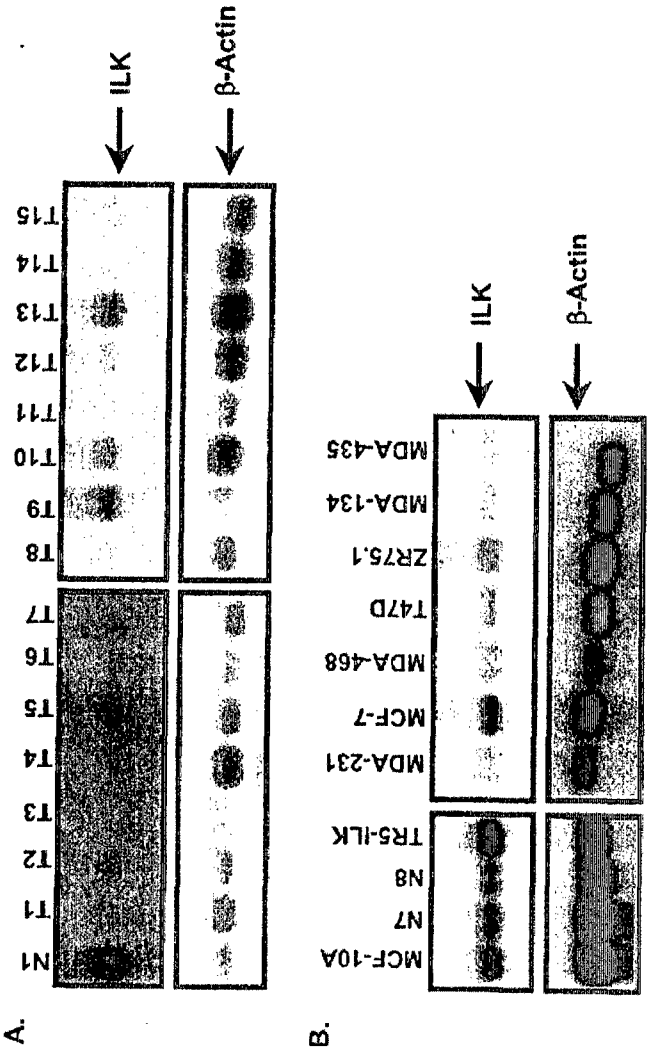

ILK
β-Actin
T15
T14
T13
T12
T11
T10
T9
T8
T7
T6
T5
T4
T3
T2
T1
N1
A.
ILK
β-Actin
MDA-435
MDA-134
ZR75.1
T47D
MDA-468
MCF-7
MDA-231
TR5-ILK
N8
N7
MCF-10A
B.

FIGURE 6

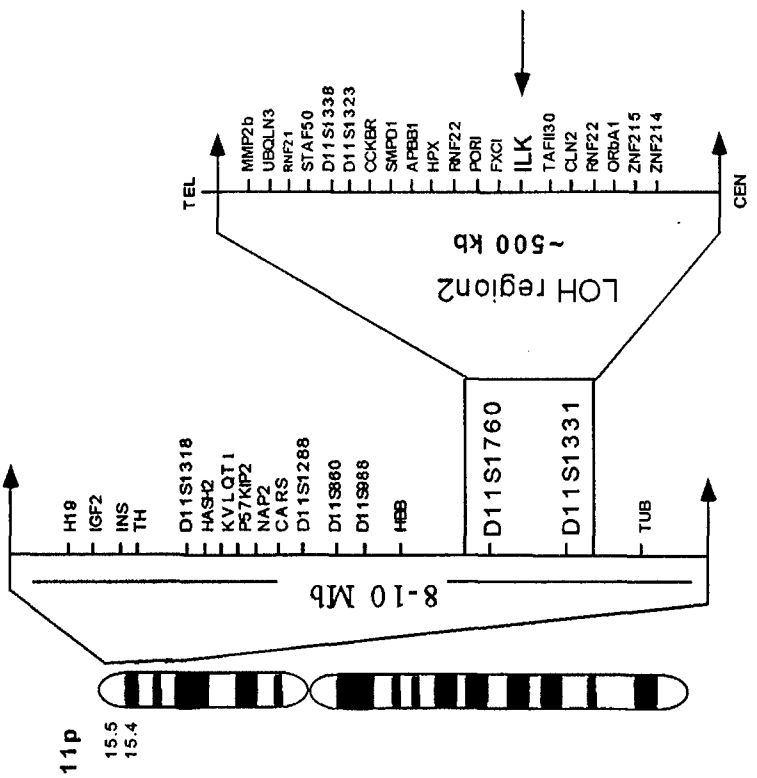

11p
15.5
15.4
8-10 Mb
H19
IGF2
INS
TH
D11S1318
HASH2
KVLQT1
P57KIP2
NAP2
CARS
D11S1288
D11S860
D11S988
HBB
D11S1760
D11S1331
TUB
LOH region2
~500 kb
TEL
CEN
MMP2b
UBQLN3
RNF21
STAF50
D11S1338
D11S1323
CCKBR
SMPD1
APBB1
HPX
RNF22
PORI
FXCI
ILK
TAFII30
CLN2
RNF22
ORbA1
ZNF215
ZNF214

FIGURE 7

A model for the role of ILK in metastasis of human breast cancer cells MDA - MB - 435

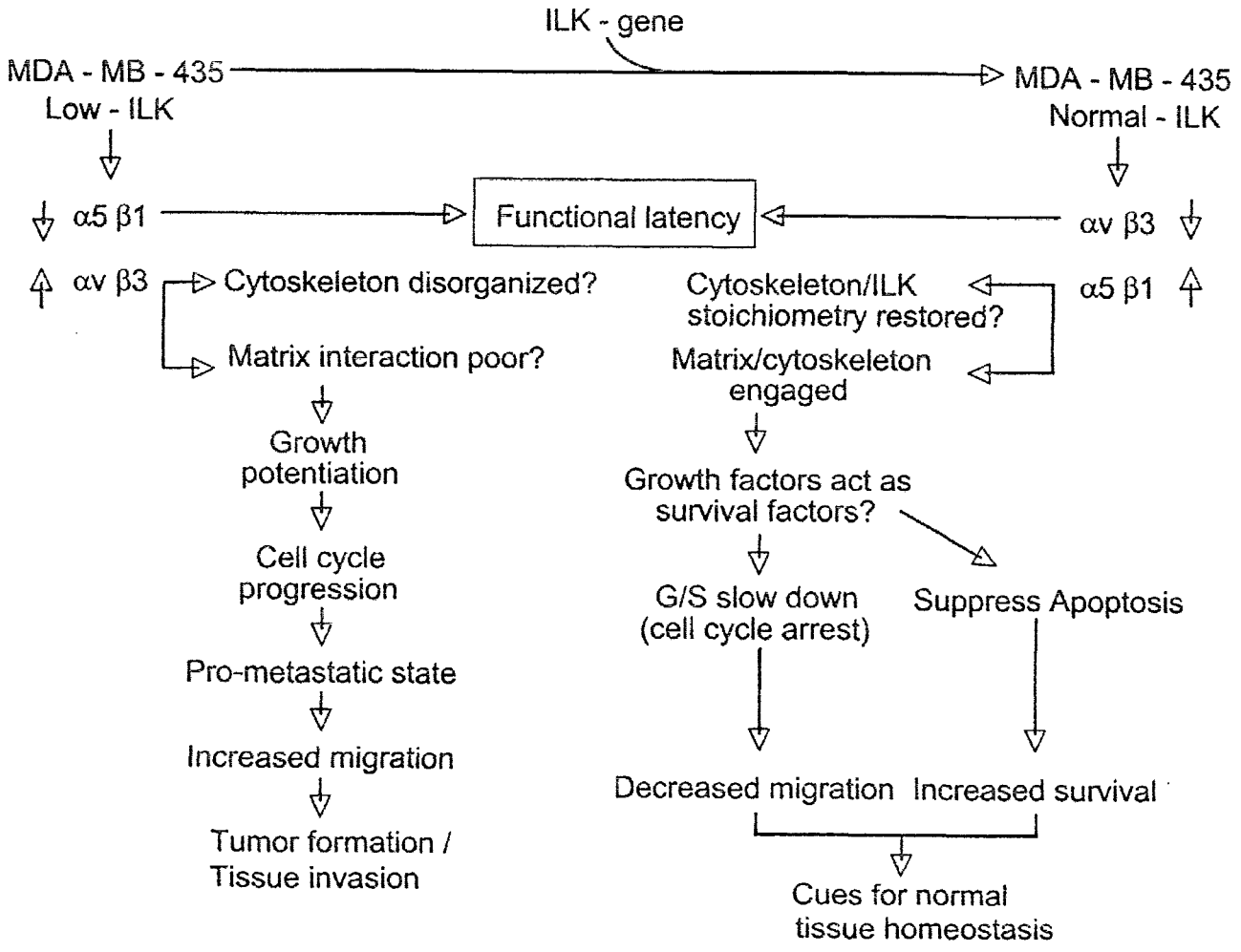


FIGURE 8

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SUPPRESSION OF MALIGNANT GROWTH OF HUMAN BREAST CANCER CELLS BY ECTOPIC EXPRESSION OF *INTEGRIN-LINKED KINASE*

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Allelic loss at the short arm of chromosome 11 is one of the most common and potent events in the progression and metastasis of breast cancer. Here, we present evidence that the *Integrin-Linked Kinase (ILK)* gene maps to the commonly deleted chromosome 11p15.5 and suppresses malignant growth of human breast cancer cells both *in vitro* and *in vivo*. *ILK* is expressed in normal breast tissue but is downregulated in metastatic breast cancer cell lines and in advanced breast cancers. Transfection of wild-type *ILK* into the MDA-MB-435 mammary carcinoma cells potently suppressed their growth and invasiveness *in vitro* and reduced the cells' ability to induce tumors and metastasize in athymic nude mice. Conversely, expression of the ankyrin repeat or catalytic domain mutants of *ILK* failed to suppress the growth of these cells. Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell-cycle progression in the G1 phase and by modulating the levels of integrins. These findings directly demonstrate that *ILK* deficiency facilitates neoplastic growth and invasion and suggest a novel role for the *ILK* gene in the suppression of tumor metastasis.

Key words: *ILK; gene transfer; breast cancer; immunohistochemistry; integrins; cell-cycle arrest; nude mouse assay*

Genetic alterations that occur in breast cancer are believed to be of importance for initiation as well as progression of the disease. These genetic alterations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis and genetic stability. The genetic abnormalities most frequently observed in breast tumors are amplification of proto-oncogenes *(MYC, ERBB2* and *CCND1),* mutations of *TP53* and loss of heterozygosity (LOH) on chromosomes 3p, 6q, 7q, 8p, 9p, 11, 13q, 17, 18q and 22q.[1,2] Metastatic phenotypes have been linked to such genes as *NME1* (17q), *CDH1* (16q), *BRMS1* (11q) and *KISS1* (1q).[1,3–5] LOH analyses have defined regions of deletion associated with metastasis on chromosomes 3p21, 15q14, 16q22 and 11p15.[2,6]

Frequent genetic alterations on chromosome 11p15 suggest a crucial role for this region in breast[6,7] and other adult[8–12] and childhood cancers.[13–17] More recently, we have mapped 2 distinct regions on chromosome 11p15.5 that are subject to LOH during breast tumor progression and metastasis.[6] LOH at region 1 correlated with tumors that contain ductal carcinoma *in situ,* suggesting that the loss of a critical gene in this region may be responsible for early events in malignancy. LOH at region 2 correlated with a more aggressive tumor and an ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Although considerable advances have been made in the fine-mapping of chromosome 11p15.5, the tumor/metastasis suppressor gene(s) encoded by this region has evaded identification.

Integrin-linked kinase (ILK) is an intriguing serine/threonine kinase that has been implicated in integrin-, growth-factor- and Wnt-signaling pathways.[18] It binds to the cytoplasmic domains of β1 and β3 integrins and mediates the downstream signaling events in integrin function.[19] Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize.[20] In *Drosophila*, the absence of *ILK* function causes defects similar to loss of integrin adhesion, and *ILK* mutations cause embryonic lethality and defects in muscle attachment.[21] Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene in tumor/metastasis suppression has not been evaluated. We have therefore analyzed the effect of *ILK* expression on the *in vitro* and *in vivo* tumor growth and invasion of human mammary carcinoma cells.

MATERIAL AND METHODS

Mapping of ILK *to LOH region 2*

Two YAC clones (847a12 and 696H10), a BAC clone (BAC 1760) and a PAC clone (PAC1331) overlapped the LOH region 2 and were used to construct a genomic contig across this the approx. 336 kb LOH region 2 (data not shown). The contig was assembled by mapping of STSs and polymorphic markers. *ILK* was mapped to this region using the primers (5′-TGGAACCCTGAACAAA-CACT-3′ and 5′-AGTCCCTGCTCTTCCTTGTA-3′).

Cell lines and cell cultures

MDA-MB-435 is an estrogen receptor- and progesterone receptor-negative, metastatic, ductal human breast carcinoma cell line derived from a 31-year-old female.[22] It has a heterogeneous chromosome complement of from 50–58 chromosomes, including several derivative marker chromosomes. The cell line readily forms tumors when injected into mammary fat pads (mfp) of nude mice, and macroscopic metastases to lungs and regional lymph nodes can be identified 15–18 weeks postinoculation. This pattern closely parallels clinical observations. Therefore, this cell line was chosen for our studies. MDA-MB-435 cells were the generous gift of Dr. Janet Price (University of Texas M.D. Anderson Cancer Center, Houston, TX). Human breast cancer cells MCF-7, T47D, MDA-MB-231, ZR75-1, MDA-MB-134, MDA-MB-468 were obtained from the American Type Culture Collection (Rockville, MD). All human breast cancer cell lines were maintained in DMEM-F12 medium supplemented with 10% fetal calf serum and no antibiotics. The neomycin-resistant ILK transfectants were maintained in DMEM-F12 containing 300 μg/ml G418 (Life Technologies, Gaithersburg, MD). All cell lines were free of *Mycoplasma* sp. contamination as determined by a PCR-based test (Pan Vera, Madison, WI).

Northern blotting, immunoblotting and immunohistochemistry

Primary tumor and adjacent normal breast tissue samples were obtained from breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). All tumor samples described in this article were determined to contain LOH at 11p15.5 region. Samples of these tumors and corresponding noninvolved tissue

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from each patient were collected at the time of surgery, snap-frozen and transferred to −80°C. Clinical and histopathologic features of the tumors were determined by the Pathology Department at CCF. An initial cryostat section was stained with H&E to determine the proportion of contaminating normal tissue in the tumor.

Total RNA from cell cultures and tumor tissues was isolated using an RNeasy Kit® (Qiagen, Chatsworth, CA). Total RNA (20 μg) was size-fractionated in a 1% agarose gel containing 2.2 M formaldehyde. After transfer and UV-cross-linking, the nylon membranes were probed with a full-length ^{32}P-labeled *ILK* cDNA. The band intensities on autoradiographs were quantitated by densitometry within linear range of signal and normalized to ribosomal 18S RNA levels. All the immunohistochemical determinations were performed on representative samples snap-frozen in liquid nitrogen and stored at −80°C until sectioning. Cryostat sections (4–6 μm thick) of tumor blocks were deparaffinized with xylene, rehydrated and microwaved for 10 min in 10 mM citrate buffer (pH 6.0). MDA-MB-435 cells were grown on sterile microscope coverslips (Fisher Diagnostics, Middletown, VA). The coverslips with MDA-MB-435 cells and slides containing tissue sections were fixed in 4% paraformaldehyde solution for 12 min, washed in PBS, preincubated with 10% Triton X-100 in PBS and incubated at room temperature with an affinity-purified rabbit polyclonal Anti-ILK IgG preparation (Upstate Biotechnology, Lake Placid, NY) for 1 hr. After being washed with PBS, bound antibodies were visualized using rhodamine-conjugated goat anti-rabbit IgG secondary antibody. Sections were visualized by fluorescence microscopy. The optimal concentration of primary and secondary antibody was determined by titration and ranged from 1:50–1:200. For negative controls, in all instances, we used non-specific IgG as the primary antibody.

For ILK immunoblotting, 5 μg of whole cell lysates (1% N-40, 0.5% deoxycholate) were separated by 10% SDS-PAGE, transferred to nylon membrane (PVDF, BioRad, Hercules, CA) and probed with an affinity-purified rabbit polyclonal Anti-ILK antibody.

Construction and transfection of wild-type and mutant forms of ILK

A partial *ILK* cDNA clone (EST bb36h09.y1, Invitrogen Corp., Carlsbad, CA) was used to obtain a full-length clone from a human placental cDNA library and its identity confirmed by sequence analysis and database comparison. The *ILK* catalytic domain mutant E359K was created as described earlier.[18] Briefly, mutations were introduced into wild-type *ILK* cDNA with the Promega Altered Sites II (Promega, Madison, WI). A mutant oligomer (with the altered nucleotide underlined) was used to change glutamic acid at position 359 to lysine (E359K, 5′-CTGCAGAGCTTT-GGGGGCTACCCAGGCAGGTG-3′). To create the ankyrin repeat deletion mutant Δ ANK (residues 165–451), the 858 bp fragment of *ILK* cDNA lacking the NH2-terminal ankyrin repeat domain was PCR amplified from the wild-type *ILK* cDNA. The mutant clones were confirmed by dideoxy sequencing. The full-length and mutant *ILK* cDNA inserts were cloned in the eukaryotic expression vector pIRES2-EGFP (Clontech, Palo Alto, CA). MDA-MB-435 cells were transfected with plasmid vector alone or containing the cDNA for *ILK* using LipofectAMINE reagent (Life Technologies). Briefly, cells were grown to approx. 60% confluence in 6 cm tissue culture dishes, rinsed twice with serum-free medium, overlaid with a mixture of 5 μg of DNA and 10 μl of LipofectAMINE reagent diluted in serum-free medium and incubated at 37°C in 5% CO_2/95% air for 18 hr. The transfection medium was then replaced with fresh medium, and 36 hr later, the cells were harvested, diluted in growth medium containing 500 μg/ml G418 and split 1:30 for the selection and establishment of clonal cell lines.

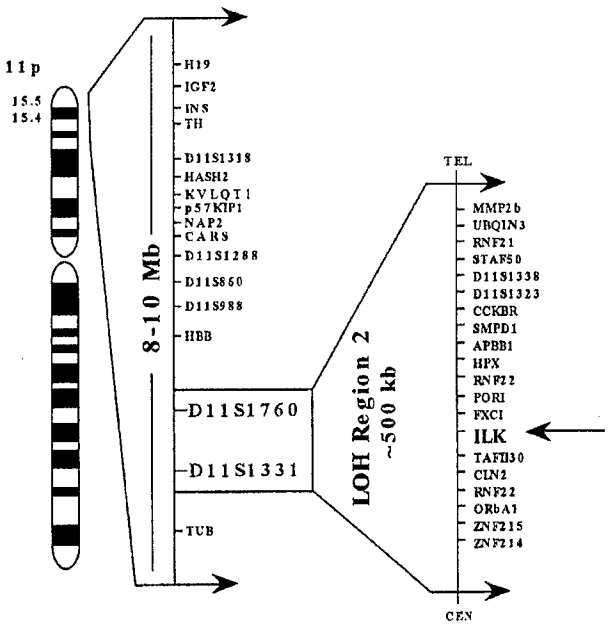

FIGURE 1 – Localization of *ILK* gene to the tumor suppressor region (LOH region 2) on chromosome 11p15.5. A transcript map of the LOH region is schematically represented with the relative location of the polymorphic markers, known genes, unigene clusters and expressed sequence tags (ESTs).

Cell growth rate, cell-cycle analysis and apoptosis assay

For growth rate analysis, untransfected, vector transfected and *ILK* transfected cells were plated at a density of 2×10^5 in DMEM-F12 plus 5% FBS medium in 24-well cluster plates. Viable and dead cells were assessed by counting with Trypan blue exclusion at Day 2, Day 4, Day 6 and Day 8. Stable *ILK* transfectants or control cells were trypsinized, and the cell numbers per dish were measured by Coulter counting and also by using a hemocytometer.

Flow cytometry was used to determine the cell-cycle distributions as described.[23] *ILK* transfected and parental MDA-MB-435 cells were washed with PBS twice, then fixed in 70% ethanol for 30 min at 4°C. The cells were treated with 1 U DNase-free RNase in 1 ml of PBS for 30 min at 37°C and finally resuspended in 0.05 mg ml propidium iodide (made as a 10× stock in PBS). Cells were analyzed by flow cytometry using a FACScan model (Becton Dickenson, San Jose, CA). Ten thousand forward scatter-gated events were collected for each sample. Fluorescence measurements were accumulated to form a distribution curve of DNA content. Fluorescence events due to debris were subtracted before analysis.

The degree of apoptosis in vector control and *ILK* transfected MDA-MB-435 cells was quantified by using a commercially available kit with fluorescein-labeled annexin V[24] (R & D Systems, Minneapolis, MN), according to the manufacturer's instructions. Samples were analyzed on a Becton Dickinson FACScan flow cytometer (a generous gift from Keck Foundation).

Cell migration assay

Cell migration assays were performed as described earlier[25] using modified Boyden chambers (tissue culture-treated, 6.5 mm diameter, 10 μm thickness, 8 μm pores, Transwell®; Costar, Cambridge, MA) containing polycarbonate membranes coated on the underside of the membrane with 10 μg/ml vitronectin in PBS. The *ILK* transfected and control cells were harvested with 0.05%

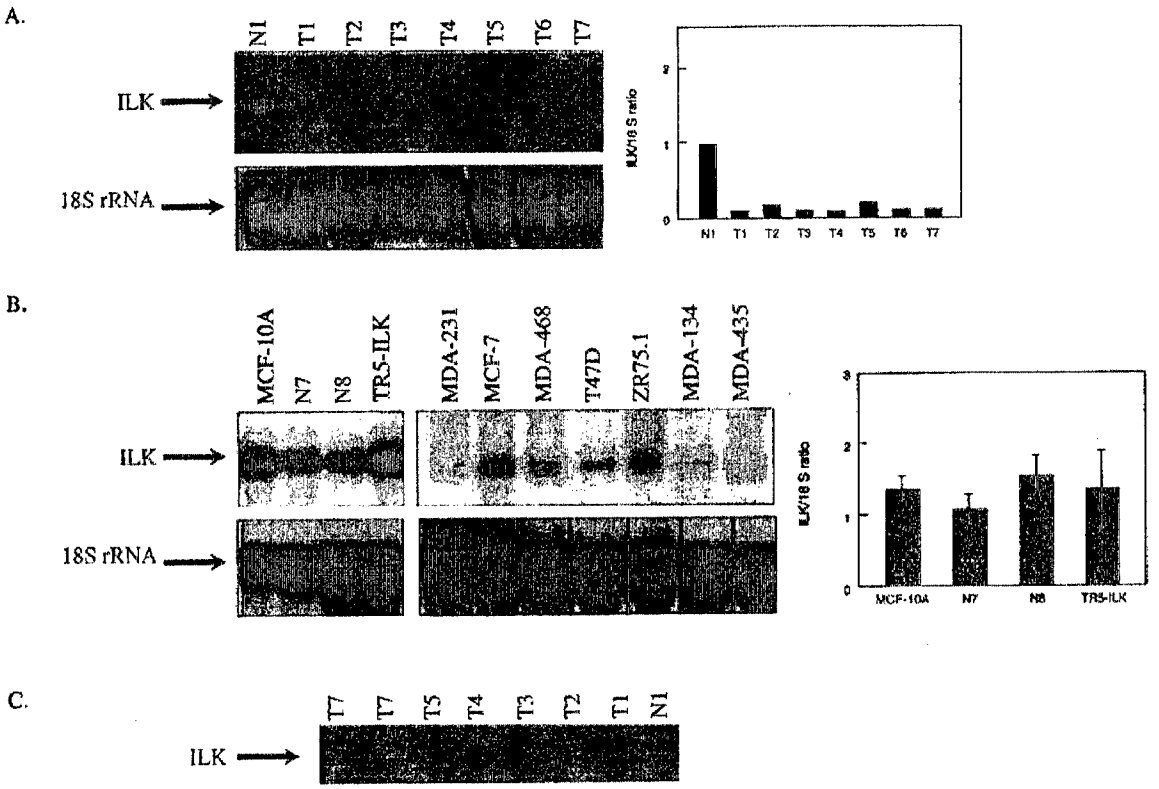

FIGURE 2 – Representative Northern blot, densitometric quantitation of the ratio of *ILK* mRNA to ribosomal 18S RNA and Western blot of *ILK* protein expression. *(a)* Total RNA was isolated from normal breast tissue (N1) and 7 invasive breast tumors (T1–T7). *(b)* Total RNA from exponentially growing nonmalignant (MCF-10A), nonmetastatic (MCF-7, T47D, ZR75.1, MDA-468 and MDA-134) and metastatic (MDA-435, MDA-231) breast cancer cell lines and normal breast tissue (N7, N8) were hybridized with ^{32}P-labeled *ILK* or 18S rRNA probe. TR5-ILK represents the ILK transfected MDA-MB-435 cell line. *(c)* ILK protein expression was determined in the whole cell lysates of normal (N1) and tumor (T1–T7) breast cell lines.

Trypsin-EDTA, washed twice with quenching medium (serum-free medium containing 5% BSA) and then resuspended in quenching medium (10^6 cells/ml). About 50,000–100,000 cells were then added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for 6 hr at 37°C in a CO_2 incubator. The nonmigratory cells on the upper membrane were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane were washed with PBS, extracted with 300 μl extraction buffer and absorbance determined at 560 nm. All values have had background subtracted, which represents cell migration on membranes coated with BSA (1%). Each determination represents the average of 3 individual wells, and error bars represent the standard deviation (SD).

Analysis of cell surface integrin profiles

Fluorescence-activated cell analysis[26] was used to identify the integrin profiles on MDA-MB-435 cells in response to *ILK* expression. Monolayer cultures (60–80% confluence) ILK transfected and control cells were trypsinized and washed in culture medium. Briefly, harvested cells were divided into equal aliquots of 2.5×10^5 cells/ml in serum-free medium plus 1% BSA. After 2 washes in this medium, the cells were resuspended in 1:50 dilution of anti- αvβ3 or α5β1 specific antibody (Chemicon, Temecula, CA) in serum-free medium plus 1% BSA for 1 hr on ice. After 2 washes in serum-free medium plus 1% BSA, the cells were incubated in 1:100 dilution of $F(ab')_2$ secondary anti-goat antibody conjugated with FITC (ICN Biomedicals, Irvine, CA) in this same medium for 1 hr on ice. The cells were washed twice in PBS/0.1% BSA and resuspended in the same solution. These samples were then analyzed using a Becton Dickinson FACScan and the data analyzed using the CellQuest Software.

Tumorigenicity and metastasis assays

Cells (10^6) were injected into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice Ncr nu/nu (10–12 mice/group; Taconic Labs, Germantown, NY) as described.[27] Mice were maintained under the guidelines of NIH and the Cleveland Clinic Foundation. All protocols were approved and monitored by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. Tumors were monitored weekly after inoculation. When the mean tumor diameter reached 1.0–1.3 cm, primary tumors were surgically removed under Keta-set-Rompun anesthetic. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastases.

RESULTS

Localization of ILK *to the LOH region on chromosome 11p15.5*

The LOH region 2 extends between the markers D11S1760 and D11S1331 on chromosomal band 11p15.5 (Fig. 1).[6] We constructed a 500 kb genomic contig (data not shown) that includes the critical region between D11S1760 and D11S1331. Using a PCR-based screening method, we initially isolated PAC and BAC clones that contained D11S1760 and D11S1331 markers. The order of the genomic clones in the contig was confirmed by

mapping of STSs, ESTs, unigene clusters and known genes that were previously mapped to chromosome 11. Eleven novel transcripts and 7 previously reported genes were PCR-mapped to the critical region between D11S1760 and D11S1331. Three of the known genes, Tata box-binding protein-associated protein (*TAF II 30*),[28] Lysosomal pepstatin insensitive protease (*CLN2*)[29] and Integrin-linked kinase (*ILK*)[30] were previously mapped only at the level of cytogenetic resolution. However, with the current mapping data, we have been able to determine the precise genomic locations of these 3 genes (Fig. 1). *ILK* and *TAF II 30* are known to be in an overlapping transcription unit in a head-to-tail arrangement. The map location and its role in multiple signaling pathways make *ILK* an attractive candidate tumor/metastasis-suppressor gene.

Loss of ILK *expression in human breast carcinomas*

To determine whether *ILK* has a role in breast cancer progression, mRNA expression in normal and tumor breast epithelial cells was compared by Northern blot hybridization and densitometry quantitation normalized to ribosomal 18S RNA (Fig. 2). A single 1.8 kb *ILK* mRNA is highly expressed in all samples of normal breast epithelial cells. A representative example is shown in Figure 2 (N1). In sharp contrast, there is either complete loss or significant downregulation of *ILK* mRNA expression in invasive breast tumors (Fig. 2*a*). Comparison of *ILK* mRNA expression in a panel of well-characterized breast cancer cell lines and in the nonmalignant breast epithelial cell line MCF-10A is shown in Figure 2*b*. *ILK* mRNA expression in MCF-10A is comparable to the expression in normal breast tissue (N7, N8) (Fig. 2*b*). However, there is a 2-5 fold downregulation of *ILK* mRNA expression in the breast cancer cell lines MCF-7, T47D, ZR75.1, MDA-468, MDA-134, MDA-231 and MDA-435 (Fig. 2*b*). Immunoblot analysis (Fig. 2*c*) indicates that the differences at the RNA levels are translated to differences at the protein level. The amount of immunoprecipitable ILK from NP-40 lysates was barely visible in 5 of 7 tumor samples compared to normal breast tissue.

To further confirm these observations, ILK protein expression was also examined using indirect immunofluorescence microscopy in frozen samples of 20 normal and corresponding pathologic human breast tissue samples. Figure 3 shows 4 representative examples. Immunohistochemical staining of normal breast tissue with ILK-specific primary antibody and rhodamine-labeled secondary antibody shows specific staining of the mammary epithelial cells surrounding the lumen in normal breast tissue from breast cancer patients. ILK expression is particularly intense in epithelial cells both within large ducts and within terminal duct lobular units but not in the stromal compartment. Incubation with purified nonspecific rabbit immunoglobulin IgG did not result in any positive staining of the normal epithelium of the breast (control). The normal breast tissue from 4 representative patients were positive (3N, 12N, 6N and 10N), whereas ILK expression was nearly completely lost in the 4 corresponding infiltrating ductal carcinomas (3T, 12T, 6T, 10T) (Fig. 3). These data show that ILK production by breast tumor cells correlates inversely with tumorigenicity and metastatic potential.

We observed a striking clinical correlation between loss of ILK expression in breast tumors and lymphatic invasion (data not shown). These tumors revealed a significantly higher incidence of metastasis to regional lymph nodes ($p = 0.01$) than tumors that expressed ILK. However, loss of ILK expression did not correlate significantly with ER/PR or p53 status or with any other critical parameters.

All breast tumor samples described in Figures 2 and 3 have previously been identified to contain LOH at the 11p15.5.[6] Allelic loss results in the reduction of gene dosage and thus may result in decreased expression. However, as seen in Figure 2, all tumors have LOH for 11p15.5 and yet only some tumors show complete loss of ILK expression. Therefore, intragenic mutations or epigenetic mechanisms might contribute to the biallelic silencing of the ILK gene in breast tumors. These mechanisms are currently under investigation.

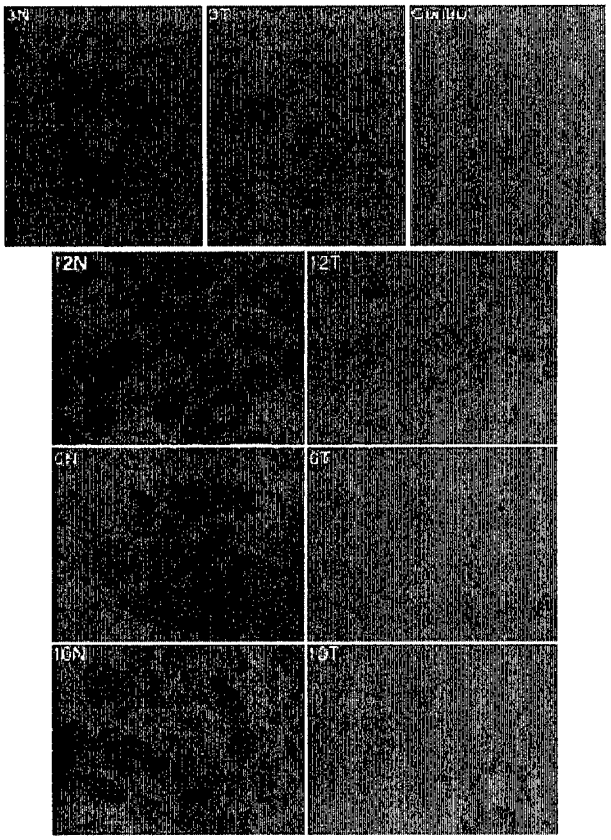

FIGURE 3 – Immunohistochemical detection of *ILK* expression in normal breast tissues (3N, 12N, 6N, 10N) and corresponding invasive ductal carcinomas (3T, 12T, 6T, 10T). Normal ducts were positive for *ILK* expression, whereas the invasive ductal carcinomas expressed little or no *ILK*. Control-Normal tissue minus primary antibody.

ILK *suppresses cell growth in human breast carcinoma cells*

The inverse correlation between *ILK* expression and tumorigenicity suggested the hypothesis that elaboration of *ILK* by tumor cells into their environment may exert an inhibitory effect. To test this hypothesis, we transfected the human breast carcinoma cell line MDA-MB-435 with the *ILK* cDNA. This cell line synthesizes very low levels of *ILK* compared to normal mammary epithelial cells (Fig. 2*b*) and can be injected into the mammary fat pad of nude mice to provide an orthotopic model system for human breast cancer tumorigenicity and metastasis. The MDA-MB-435 cells were transfected with a mammalian expression vector pIRES-EGFP containing full-length *ILK* cDNA under control of the CMV promoter. A total of 4 stable clones expressing different levels of *ILK* have been established. Comparison of mRNA expression by Northern blot analyses revealed that the clones TR4 and TR5 expressed slightly higher levels of *ILK* mRNA compared to the clones TR2 and TR3 (Fig. 4*b*). Based on Northern blot analysis, *ILK* expression in clone TR5 is comparable to the expression in the nonmalignant breast epithelial cell line MCF-10A and to the expression in normal mammary epithelial cells (Fig. 2*b*), suggesting that ILK expression is restored to normal levels upon transfection. The expression of *ILK* in empty vector controls (data not shown) is comparable to untransfected MDA-MB-435 cells (UT). ILK protein levels in transfected (TR3-TR5) and untransfected

cells (UT) were determined by indirect immunofluorescence and Western blotting. The ILK protein levels in these cells are restored to normal breast tissue levels (Fig. 4*c*), and the expressed ILK protein is localized in the cytoplasm (Fig. 4*a*, panel c). Most strikingly, corresponding to the low levels of *ILK* mRNA (Fig. 2*b*), the highly metastatic MDA-MB-435 cell line exhibited very little detectable ILK protein (Fig. 4*a*, panel b, and 4*c*).

To determine whether *ILK* expression had any effect on the growth properties of the MDA-MB-435 cells, we determined the growth kinetics of the clones TR3 and TR5. *ILK* expression causes the MDA-MB-435 cells to grow to a low saturation density (Fig. 5*a*), and there is substantial growth suppression of the TR5 clone compared to untransfected MDA-MB-435 cells. The growth suppression of the transfectants was *ILK* concentration dependent with TR5 (high-expressing clone) growing to a lower saturation density than TR3 (low-expressing clone). Furthermore, the growth rate of TR5 was decreased by approx. 40% with a cell doubling time of 96 hr compared to the growth rate of cells transfected with vector alone or untransfected cells, which had a doubling time of 48 hr. However, there were no remarkable changes in cell morphology.

The ability of ILK to suppress growth could be either due to a nonspecific lethal effect of protein overproduction or due to a more specific effect on cell proliferation. Alternatively, antisense interference of *ILK* mRNA with the *TAF II 30* may also be responsible, since the overlapping *ILK* and *TAF II 30* transcription units are in a head-to-tail arrangement. To further establish a link between ILK protein function and growth suppression, we tested the growth kinetics of 2 *ILK* variants. *ILK* contains 4 ankyrin repeats at the NH2-terminus[18] that participate in protein-protein interactions important for integrin-, growth-factor- and Wnt-mediated signaling. First, a deletion mutant, Δ ANK lacking this domain was constructed. In addition, the residue E359 has been shown to be

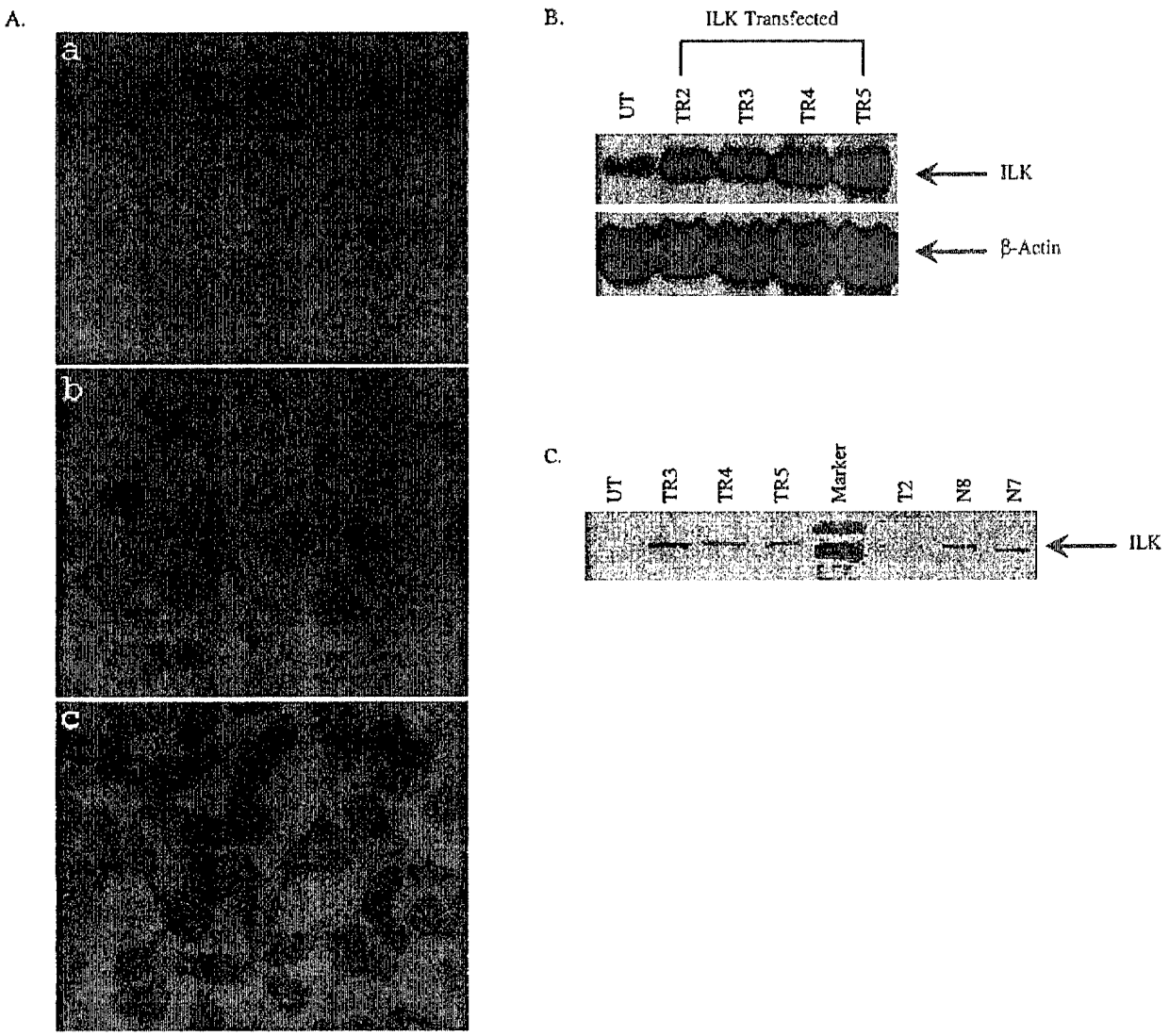

FIGURE 4 – MDA-MB-435 cells were transfected with pIRES-EGFP vector containing full-length *ILK* cDNA, and 4 stable clones were isolated. *(a)* Immunohistochemical analysis of *ILK* expression in the MDA-MB-435 cells before (panel b) and after (panel c) transfection (stable clone TR5-*ILK*). (Panel a) No primary antibody control. *(b)* Northern blot analysis of parental (UT) and *ILK* transfected MDA-MB-435 cells. TR2, 3, 4 and 5 represent stable *ILK* expfessing clones. mRNA expression was determined by hybridization with ^{32}P-labeled *ILK* probe. β-actin expression serves as control. *(c)* ILK protein expression was determined by Western blotting in whole-cell lysates of untransfected (UT), MDA-MB-435 cells transfected with *ILK* cDNA constructs (TR3, TR4 and TR5), normal (N7, N8) and tumor (T2) breast cells.

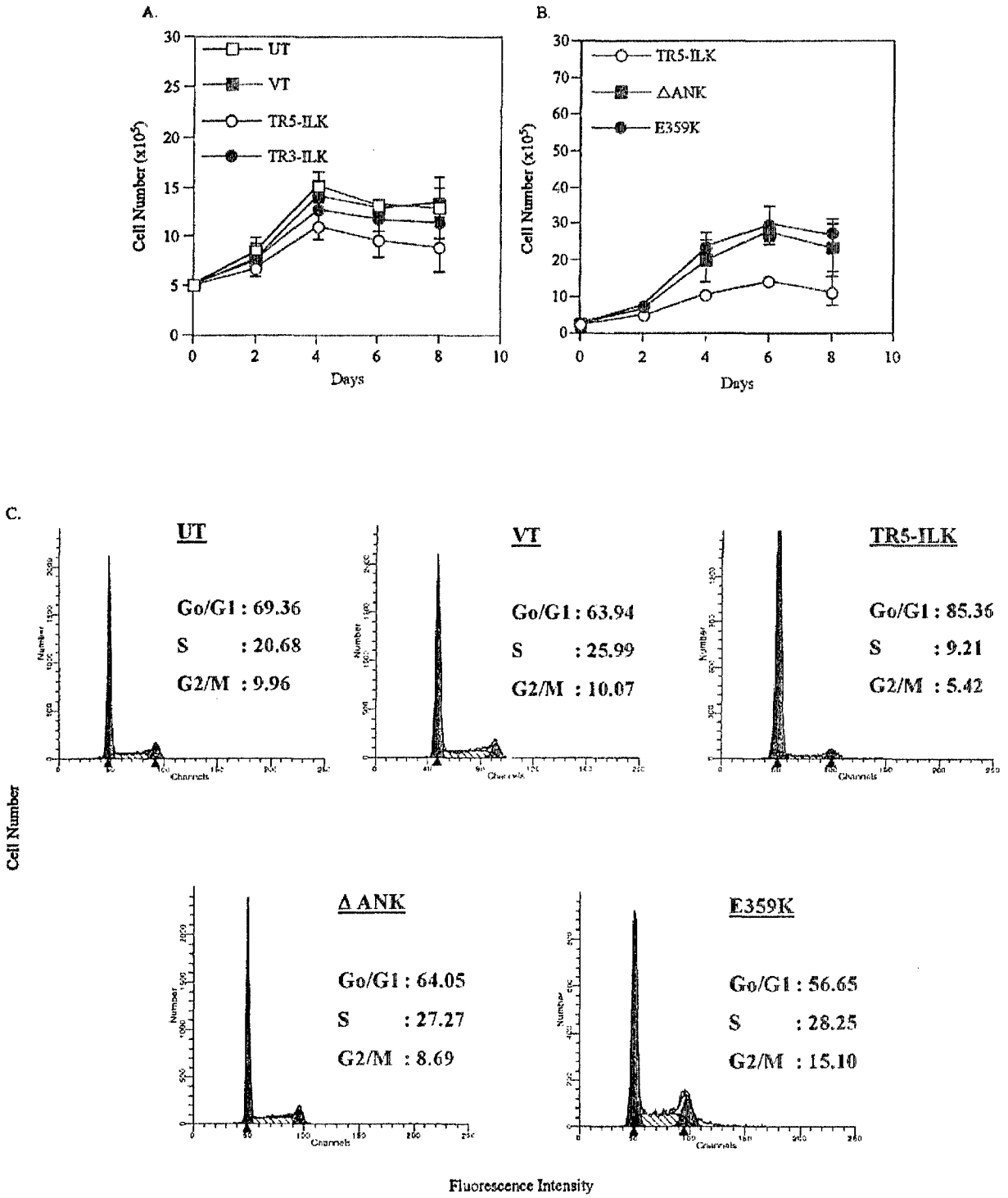

FIGURE 5 – Growth effects of wild-type and mutant alleles of *ILK* in MDA-MB-435 breast cancer cells. The MDA-MB-435 cells were transfected with either full-length *ILK* cDNA, *ILK* mutant ΔANK, *ILK* mutant E359K or eukaryotic expression vector, and stable clones were obtained. *(a)* Growth rates of 2 stable *ILK* expressing MDA-MB-435 cell clones (TR3-*ILK* and TR5-*ILK*) that contain full-length *ILK* cDNA compared to a stable clone containing empty vector (VT) and untransfected MDA-MB-435 cells (UT). *(b)* Growth rates of *ILK* mutants ΔANK and E359K compared to the wild-type *ILK* expressing clone TR5-*ILK*. The means of 3 independent experiments are shown. Bars represent S.E. *(c)* Cell-cycle analysis by propidium iodide staining in MDA-MB-435 cells (UT), transfected with empty vector (VT), with full-length *ILK* cDNA (TR5-*ILK*) or with the *ILK* mutants ΔANK and E359K. The regions between the vertical lines from left to right represent cells in G0/G1, S and G2/M, respectively.

essential for *ILK* function.[18] We therefore constructed an *ILK* point mutant (E359K) in which the highly conserved Glu359 within the *ILK* catalytic domain was substituted with lysine. Whether the residue E359 is responsible for kinase activity is unclear;[31] however, it is clear that E359K mutation interferes with ILK protein function. In transfected clones, the level of expression of mutant ILK proteins was comparable to that of wild-type ILK. The growth rates of the stably transfected *ILK* mutant clones Δ ANK and E359K compared to the *ILK* transfectant TR5 are shown in Figure 5*b*. As discussed above, expression of the wild-type *ILK* strongly inhibited growth of the MDA-MB-435 cells. In contrast, both the Δ ANK and E359K mutants lost their capacity to suppress the growth of the MDA-MB-435 cells (Fig. 5*b*), arguing against a nonspecific effect of protein overproduction as well as antisense interference with *TAF II 30* transcription unit.

Expression of ILK *in MDA-MB-435 cells leads to a G1 cell-cycle arrest*

The observed growth suppression by *ILK* could be caused by either increased apoptosis or inhibition of cell proliferation. To investigate the mechanisms underlying the growth suppression by *ILK* expression, we studied apoptosis by fluorescence-activated cell sorting (FACS) analysis of Annexin-V stained *ILK* and vector transfectants. There was no increase in the rate of apoptosis in *ILK*-expressing cells compared to vector transfectants (data not shown). Therefore, programmed cell death does not seem to account for the growth suppression of *ILK* transfected cells.

To test for cell-cycle regulation by ILK, propidium iodide-stained MDA-MB-435 clones were analyzed by flow cytometry. Expression of ILK increased the number of cells in G0/G1 from 64% to 85% (Fig. 5*c*, VT and TR5-ILK) and decreased inversely the number of cells in S and G2/M phase from 26% and 10% to 9% and 5% (Fig. 5*c*, VT and TR5-ILK). In contrast, the cell-cycle profiles of the 2 ILK variants Δ ANK and E359K were very similar to the parental MDA-MB-435 cells. These results indicate that ILK growth suppression results from G1 cell-cycle arrest. The accumulation of cells in the G0/G1 phase of the cell cycle suggests arrest predominantly at the G1/S boundary. ILK expression does not induce cell death or apoptosis but induces a very pronounced growth arrest with 85% of the cells in G0/G1, a property that is the hallmark of growth/tumor suppressors.

ILK *suppresses the invasive phenotype of human breast carcinoma cells*

Cell migration on vitronectin *in vitro* has been linked to the metastatic capacity of tumor cells *in vivo*.[32,33] To examine the effects of *ILK* expression on breast cancer cell invasion, the ability of vector and *ILK* transfected MDA-MB-435 cells to degrade and invade vitronectin-coated polycarbonate membrane was investigated. As shown in Fig. 6*a*, a significant reduction in invasive potential was noted in the ILK-expressing clone TR5 compared to vector transfected MDA-MB-435 cells (VT) (Fig. 6*a*). Cell invasion through membranes coated with vitronectin is decreased by 60% in MDA-MB-435 cells expressing *ILK* compared to vector transfected MDA-MB-435 cells. In contrast, the 2 ILK variants Δ ANK and E359K have no significant effect on cell invasion under identical conditions (Fig. 6*a*).

In fact, there is a slight increase in invasive potential of the variant clones (Δ ANK and E359K), suggesting a dominant-negative effect, perhaps due to inhibition of endogenous ILK in the MDA-MB-435 cells. These results indicate that *ILK* expression abates extracellular matrix invasion of tumor cells *in vitro*, one of the hallmarks of tumorigenicity and transformed cell growth.

Cell adhesion, migration and invasion are controlled by the levels of integrins and by the amount of fibronectin matrix around the cell.[20] Because the α5β1 and αvβ3 integrins have been implicated in the regulation of angiogenesis, tumor cell migration, invasion and metastasis, we speculated that *ILK* might regulate cell migration via alteration of the cellular composition of integrins.

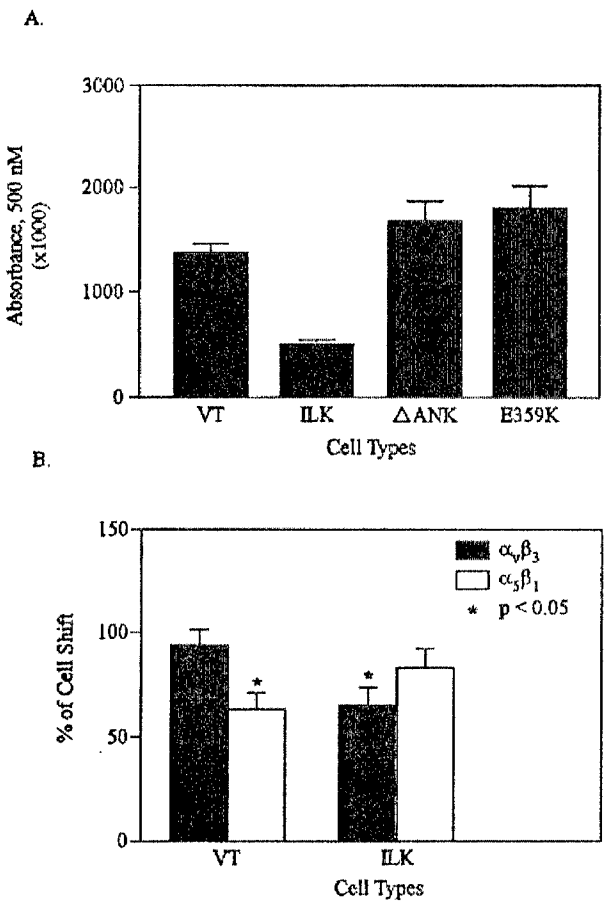

FIGURE 6 – (a) Cell invasion assay of MDA-MB-435 cells transfected with vector (VT), full-length *ILK* and its variants (ΔANK, E359K). Cell invasion through vitronectin was analyzed using a modified Boyden chamber. Cells that invaded to the lower surface of the membrane were lysed and absorbance determined at 560 nm. *(b)* Flow cytometric analysis of α5β1 and αvβ3 integrins expressed on the surface of *ILK* transfected and parental MDA-MB-435 cells. The relative fluorescence intensity of cells stained with α5β1 and αvβ3 antibodies is represented as percentage of cell shift. Bars represent S.E.

Using specific antibodies against these integrins in flow cytometry analysis, we compared integrin expression patterns in relation to the *ILK* expression status. The results are shown in Figure 6*b*. The *ILK* transfected cells demonstrated a 22% increase in levels of the growth-suppressing integrin α5β1 and a 31% decrease in levels of the growth-promoting integrin αvβ3 compared to the control cells. The changes in levels of αvβ3 and α5β1 expression in *ILK* transfected cells, although relatively moderate in comparison to control cells, nonetheless are highly significant. Collectively, these observations suggest that *ILK* reduces the invasive potential of MDA-MB-435 cells by altering their integrin profiles, which changes their ability to perceive and interact with their extracellular environment.

ILK *suppresses tumor formation and metastasis in nude mice*

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet not all of the cellular growth properties commonly associated with the cellular state *in vitro* are required for neoplastic growth *in vivo* and vice versa. Therefore, loss of tumorigenicity under expression of *ILK in vivo* would be a critical test to substantiate the growth

suppressor function of *ILK*. The mammary carcinoma cell line MDA-MB-435 forms tumors at the site of orthotopic injection, metastasizes in nude mice and closely resembles the course of human breast cancer.[34] To investigate whether *ILK* expression affected tumor formation in nude mice, 2 different *ILK* transfectant clones (TR5-ILK and TR3-ILK) and 2 vector controls were inoculated into the subaxillary mammary fat pads of 4–6-week-old athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. All MDA-MB-435 vector transfectants were already palpable 7 days after injection. Subsequently, the tumors of vector transfectants grew steadily, attaining mean volumes of 3.0 cm^3 (mean ± S.D.) at 15 weeks (Fig. 7*a,b*). In contrast, only 2 of 12 mice injected with ILK transfectants developed tumors. The tumor growth of ILK transfectants was significantly slower than that of control transfectants ($p < 0.005$, Fisher variance analysis). At sacrifice (15 weeks), the *ILK* tumors reached a mean volume of only 0.45 cm^3 (mean ± S.D.), which was significantly smaller than control tumors ($p < 0.001$, Student's *t*-test). Vector transfected MDA-MB-435 cells developed an average of 12–24 lung metastases per mouse (Fig. 7*c*). Additional tumor masses were present in central venous blood vessels, the diaphragm and lymph nodes of vector transfectants (data not shown). In contrast, with the ILK transfectants, only 1 of the 2 animals that developed tumors exhibited a single metastatic colony in the lung. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). These results clearly demonstrate that the expression of *ILK* in human MDA-MB-435 breast carcinoma cells significantly suppresses tumorigenicity and metastatic ability in athymic nude mice.

DISCUSSION

Growth-inhibitory functions of ILK

Our study reveals that expression of *ILK* potently suppresses *in vitro* growth and invasiveness and *in vivo* tumorigenicity of the human mammary carcinoma cells. The MDA-MB-435 cells are a model for deficient *ILK* protein expression, and transfection of the *ILK* gene is designed to restore this deficiency. The growth-suppression activity requires a functional *ILK* protein, since expression of wild-type *ILK*, but not the ankyrin repeat or the catalytic domain mutants, resulted in growth suppression of MDA-MB-435 cells. These results suggest a possible role for *ILK* in the suppression of tumor growth and metastasis and directly implicate its loss in processes regulating the malignant phenotype in human breast cancer. *ILK* seems to play a dual role in the MDA-MB-435 model system. First, it regulates cell-cycle progression at the G1/S boundary, and second, it modulates the levels of integrins, trans-

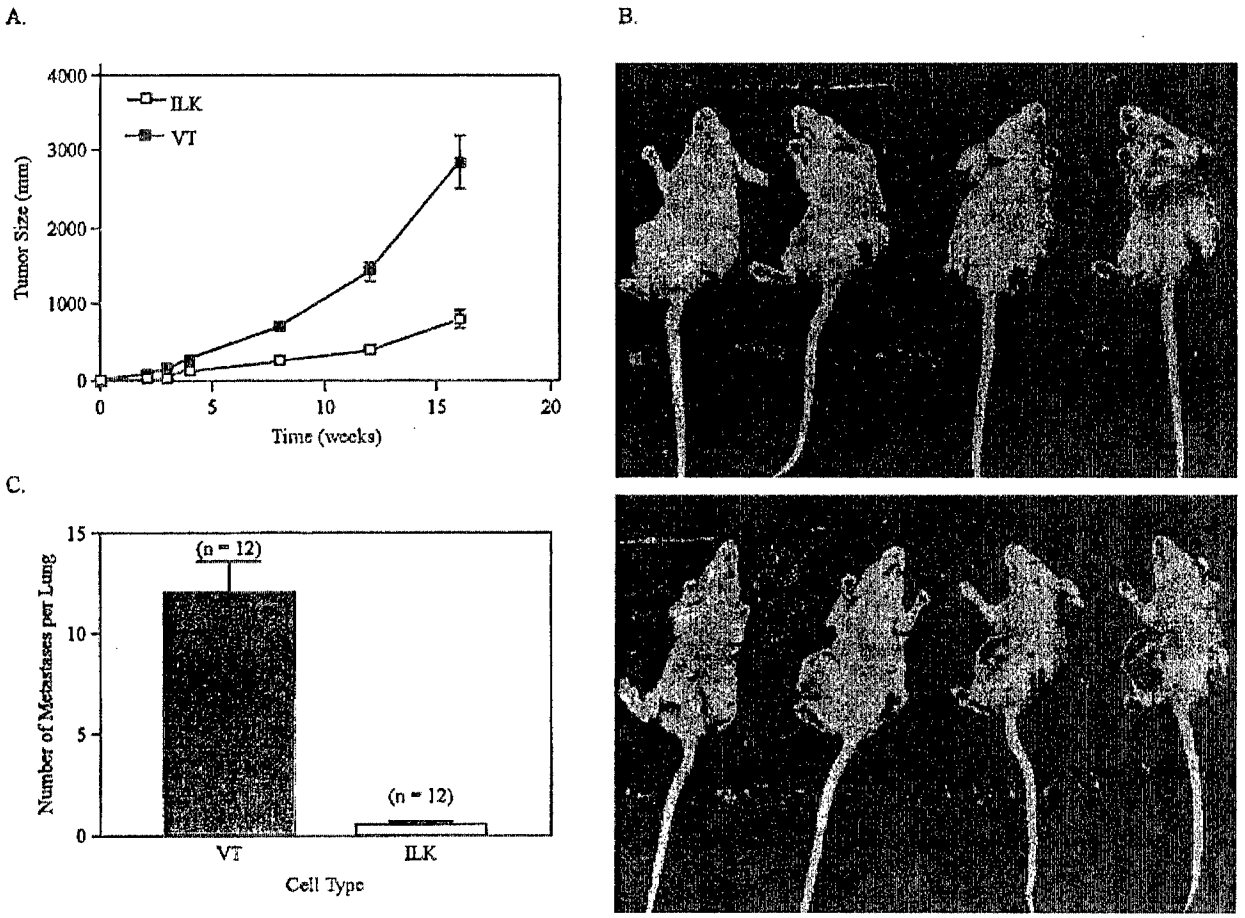

FIGURE 7 – *(a) In vivo* tumor growth of *ILK* transfected and vector transfected MDA-MB-435 cells in mammary fat pads of athymic nude mice. Each point represents the mean ± S.E. of tumors. *(b)* An amount of 5×10^5 cells of *ILK* transfected (top panel) or vector transfected (bottom panel) MDA-MB-435 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 15 weeks, at which time the mice were photographed and sacrificed. *(c)* Lung colony formation in athymic nude mice injected with vector transfected (VT) or *ILK* transfected (*ILK*) MDA-MB-435 cells. Bars represent S.E.

membrane receptors that have been shown to regulate growth, differentiation and invasiveness of cells. During this process, the neoplastic cells cease to proliferate and lose their ability to migrate through vitronectin membranes (Fig. 6) and to induce tumor growth and metastasis in nude mice (Fig. 7). Our results are consistent with earlier micro-cell-mediated chromosome transfer experiments showing that introduction of human chromosome 11 into MDA-MB-435 cells suppressed metastasis in nude mice.[35]

Our observations on the role of ILK in the highly invasive mammary carcinoma cell line differ from earlier findings of ILK overexpression in normal epithelial cells. Overexpression of ILK in normal epithelial cells (Scp2 mouse mammary epithelial cells and IEC-18 rat intestinal epithelial cells) results in anchorage-independent growth,[36] cell-cycle progression[37] and tumorigenicity in nude mice.[38] The apparent contradiction between these 2 models may be due to the cellular context in which ILK was expressed. Normal epithelial cells express optimal levels of ILK, whereas the MDA-MB-435 cells have sub-threshold ILK expression. Thus, the effects we report are due to restoration of the cellular ILK levels, whereas the results of ILK transfection in normal cells likely represent the effects of ILK overexpression. These differences could account for distinct phenotypic changes in the 2 experimental models. For instance, normal epithelial cells have a different integrin composition compared to breast cancer cell lines,[39,40] and any changes in these cells that perturbs the ratio of various integrins (such as ILK overexpression) can be expected to differently affect the fate of these cells. The $\alpha5\beta1$ integrin is expressed by the majority of epithelial cell types, including those of breast, skin, lung, gastrointestinal system and the genitourinary tracts.[39,41] Whereas the normal breast ducts and ductules express high levels of $\alpha5\beta1$ integrin,[41] the expression pattern of integrins in invasive mammary carcinoma cells is atypical, and abnormalities observed include loss, downregulation or improper localization $\alpha5\beta1$ integrin at the cell surface.[42,43] The $\alpha5\beta1$ integrin is diminished in moderately differentiated carcinoma, and its expression is markedly reduced or undetectable in poorly differentiated adenocarcinoma of the of the breast.[40–43] The $\alpha5\beta1$ integrin has been directly implicated in the growth inhibition of tumor cells.[40] In contrast, expression of the $\alpha v\beta3$ integrin positively regulates tumor cell proliferation.[40] Integrin $\alpha v\beta3$ is minimally expressed in normal epithelial cells and in normal blood vessels and is significantly upregulated within human tumors.[44] In several malignancies, tumor cells express $\alpha v\beta3$, and this expression correlates with tumor progression in melanoma, glioma and ovarian, prostate and breast cancer.[40,45] In breast, $\alpha v\beta3$ characterizes the metastatic phenotype as this integrin is upregulated in invasive tumors and in distant metastases.[45,46] Thus, the pattern of integrin expression in the tumor cell is implicated in the enhanced proliferation that is a characteristic of tumor cells. The data we have obtained with the MDA-MB-435 cells are consistent with a role for ILK in the modulation of integrin expression and integrin-regulated cellular proliferation. The MDA-MB-435 studied here were chosen for the metastatic property that easily formed tumors in nude mice. We speculate that the growth-suppression role described for ILK using this model is a general phenomenon that occurs in all cell lines in response to changes in ILK levels.

In the context of a highly invasive mammary carcinoma cell, we have demonstrated that ILK transfection causes a reversal in ratio of expression of integrin $\alpha5\beta1$ to $\alpha v\beta3$. However, it is highly likely that the levels of other integrins such as $\alpha6\beta1$ may also change either directly in response to ILK or indirectly due to changes in the availability of $\beta1$ or $\beta3$ integrins. This change is associated with a decrease in the cells' ability to transmigrate *in vitro* and metastasize *in vivo*. It is well documented that changes in how cells interact with their environment via altered, modulated or regulated integrin interaction can have dramatic and far-reaching consequences for both normal and pathologic conditions. Studies have shown that perturbations of certain integrins (by either ligation or treatment with certain anti-integrin antibodies) can generate

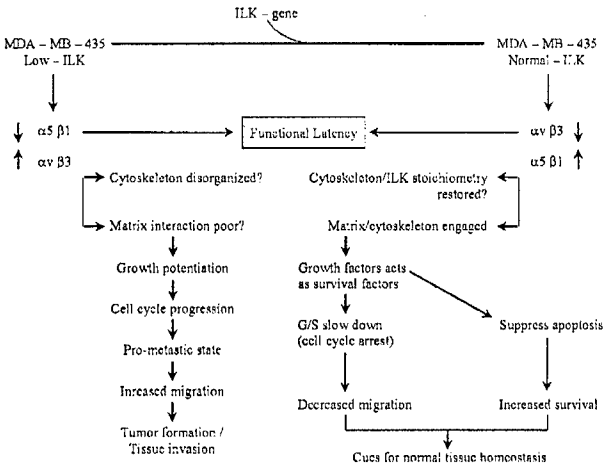

FIGURE 8 – Model for the growth and metastasis of human breast cancer cells MDA-MB 435. The highly invasive and metastatic cell line MDA-MB-435 has an abnormal integrin repertoire and altered cell matrix interaction due to loss of ILK expression. These cells become less invasive and metastatic upon *ILK* gene transfer, which restores the stoichiometry of the integrins and cytoskeletal elements. Postulated changes in intracellular regulatory events are shown.

signals which result in an increase in intracellular pH, and Ca^{2+} levels, changes in inositol lipid synthesis, tyrosine phosphorylation of $pp125^{FAK}$, activation of p34/cdc2 and cyclin A, activation of protein kinase C, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, Ras, and NF-κB.[40,47] Furthermore, the $\alpha v\beta3$ on melanoma cells has been shown to bind and localize proteolytically active MMP-2 at the cell surface which appears to facilitate cell mediated collagen degradation and directed cellular invasion.[48] Clearly, integrins are key molecules to integrate intrinsic and extrinsic events of the cellular behavior. They profoundly influence oncogenesis and the metastatic process. Thus, changes in integrin composition brought about through restoration of ILK protein expression in invasive breast carcinoma cells can be expected to bestow completely different, and even opposite effects, compared to the effects of ILK protein overexpression in normal cells.

A likely mechanism for the growth and metastasis of the breast cancer cells MDA-MB-435 is shown in Figure 8. Focal contact with the extracellular matrix is a fundamental mechanism by which cells initiate intracytoplasmic signaling in order to regulate differentiation, growth, attachment, migration, invasion and metastasis. The stoichiometry of integrins, cytoskeletal elements and ILK is probably an essential factor for the proper functioning of these cellular processes. Regulation of cellular ILK level is critical for maintaining the integrin repertoire of the cell that is essential for normal interactions between cells and the extracellular matrix. The highly invasive and metastatic cell line MDA-MB-435 has an abnormal integrin repertoire and altered cell matrix interaction due to loss of ILK expression. The increased expression of $\alpha v\beta3$ and reduced expression of $\alpha5\beta1$ integrin and perhaps changes in other integrins as well make these cells highly proliferative, invasive and metastatic both *in vitro* and *in vivo*. These cells become less invasive and metastatic upon *ILK* gene transfer, which restores the stoichiometry of the integrins and cytoskeletal elements. *ILK* downregulates $\alpha v\beta3$ and upregulates the expression of $\alpha v\beta1$ integrin and could as well affect the expression levels of other integrins not examined here. This in turn leads to G1 cell-cycle arrest, decreased migration and cues for tissue preservation. It is highly likely that the effects seen are accompanied by composite changes in many other cell surface integrins and receptors as well. In breast cancer, altered cell matrix contact due to altered integrins

has been shown to be a prerequisite for metastasis.[49] Based on this model, we speculate that *ILK* transfection in normal cells alters their integrin profile and integrin-cytoskeletal stoichiometry, leading to a decrease in cell extracellular matrix interactions. This in turn induces anchorage-independent growth,[36] cell-cycle progression[37] and tumorigenicity in nude mice.[38] Clearly, the functions of *ILK* are more complex than previously envisioned, and the divergent and often paradoxical effects mediated by *ILK* may depend on the particular cell type, cell environment and the cell-specific integrins that are activated.

In conclusion, we have shown that the loss of *ILK* expression is associated with the acquisition of a malignant breast tumor phenotype and that *ILK* may directly act as a growth suppressor, presumably by controlling cell division and by modulating the levels of integrins. The absence of the *ILK* may promote uncoordinated G1 cell-cycle progression, allowing cells to bypass the normal signaling processes regulated by growth factors and cell anchorage, leading to tumorigenesis.

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